STROMAL CHANGES IN APPARENTLY NORMAL MUCOSA OF SMOKERS AND PANCHEWERS – A MULTI-PARAMETRIC APPROACH USING HISTOPATHOLOGY, ULTRASTRUCTURE AND AUTOFLUORESCENCE

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SAVEETHA DENTAL COLLEGE, CHENNAI.

CERTIFICATE

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INTRODUCTION

Introduction

Habit is a pattern of behavior acquired through frequent repetition. Habits can be beneficial or deleterious in nature. Tobacco usage, alcohol intake or combinations of both are examples of deleterious habits. These preventable habits are associated with significant morbidity and mortality. The population should be aware of the potential consequences of prolonged exposure to tobacco and alcohol [147].

Tobacco use, including smoking of cigarettes, cigars and pipes, reverse smoking (smoking with the lit end inside the mouth), chewing of betel quid (a mixture of areca nut, slaked lime, and tobacco wrapped in betel leaf), and use of smokeless tobacco increases the risk of cancers of the upper aerodigestive tract [27, 42]. *Tobacco usage is, together with drinking alcohol, the major risk factor for oral cancer.*

Oral cancer is dominated by squamous cell carcinoma (present in 90%-95% of all oral cancers), and the role of tobacco in the development of oral squamous cell carcinoma is well recognized. The long-term prognosis is quite poor, and treatment can lead to further functional and cosmetic problems [104, 105].

Based on population-based case-control studies, cigarette smokers have risk of two to five times than that of non-smokers for developing oral cancer. The risks increase with the numbers of cigarettes smoked and the duration of smoking. Tobacco-specific N-nitrosamines, aromatic amines, and polycyclic aromatic hydrocarbons present in mainstream tobacco smoke are considered as the major carcinogens [103].

Although epidemiologic studies consistently report that, risk of oral cancer declines with the number of years of abstinence from cigarettes, it can take many years for the risks to reach those of non-smokers.

Studies have also demonstrated that drinking alcoholic beverages is associated with the development of cancers in the oral cavity [106]. When ethanol is consumed through drinking, it is metabolized primarily by class I alcohol dehydrogenase (ADH2) into acetaldehyde, an intermediate metabolite, and then it is metabolized by aldehyde dehydrogenase (ALDH2) into acetic acid in humans [107]. Acetaldehyde, a well-known carcinogen in animals, plays an important role in alcohol toxicity in humans [108].

Heavy smokers who drink alcohol heavily have an increased risk of developing oral cancer than that expected from the independent effects of smoking and alcohol intake [147].

Smokeless tobacco, in the forms of chewing tobacco and snuff, is also linked to oral cancer. Tobacco is chewed predominantly as an ingredient of betel quid or pan, which is a combination of betel leaf, areca nut, and lime. Based on analysis that excluded smokers, smokeless tobacco users experienced about four to six times the risk of oral and pharyngeal cancer than non-users. Nitrosamines are produced at relatively high levels during fermenting and curing and these products have an etiological role [104, 105].

Premalignant lesions of the oral cavity represent an important target for cancer prevention. The two most important lesions are leukoplakia and sub-mucous fibrosis.

These lesions represent clinical rather than histological diagnoses, but their importance derives from the high proportion of cases in which biopsy reveals dysplasia or even frank carcinoma. These lesions can be detected by visual inspection and are amenable for large-scale screening efforts.

Oral leukoplakia is currently defined as a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion; some of these lesions will develop into cancer. The recent classification and staging system also incorporates provisional and definitive diagnoses on the basis of histopathological features of persistent lesions lasting longer than 2 to 4 weeks, such as the size of the leukoplakia and the presence of epithelial dysplasia. There is a significant correlation between the incidence of leukoplakia and smoking [109].

Smokers with oral premalignant lesions such as leukoplakia have an annual cancer transformation rate of about 5% [110]. A case-control study, conducted by Shiu et al in Taiwan, showed that the adjusted ratio for betel nut chewing and smoking on the occurrence of leukoplakia were 17.43 and 3.22, respectively. The findings suggested that stopping smoking may reduce the number of leukoplakia cases by 36%, while elimination of betel nut and tobacco influence may prevent 62% cases of leukoplakia and 26% of cases of malignant transformation to oral carcinoma [111].

In the Indian scenario, the proportion attributed to tobacco use in the form of smoking and chewing comprises about 61-70% of cancer incidence. Among Indians, alcohol drinking does not emerge as a strong risk factor [115].

Sub-mucous fibrosis is a pre-cancerous condition, predominantly affecting buccal mucosa, retromolar area and the soft palate. Recently, an increased incidence of malignancy is noted in oral submucous fibrosis patients, particularly in people who use commercially available products. The malignant transformation of betel quid users in India is around 8%, which is quite high [115, 145].

There is increasing evidence that sub-epithelial connective tissue can modify the phenotypic expression of the overlying epithelium. Various tissue culture studies suggest that the histo-differentiation of the epithelium, including its phenotype and keratin expression could be extrinsically modified by mesenchymal fibroblasts [112, 113, 114]. *We could elucidate that the underlying connective tissue plays a pivotal role in the maintaining the integrity of overlying epithelium.*

Therefore, a study was designed with varying parameters and tools to evaluate the stromal changes in apparently normal oral mucosa of patients with or without deleterious habits. The stromal changes were then compared with leukoplakia, oral sub-mucous fibrosis, well differentiated squamous cell carcinoma, moderately differentiated squamous cell carcinoma and verrucous carcinoma.

AIMS & OBJECTIVES

AIMS AND OBJECTIVES

1. *To compare and contrast the various changes in the connective tissue among patients with the habits of smoking (n=10), pan chewing (n=10) and Controls (n=10) by using fluorescence spectroscopy.*
2. *To evaluate the connective tissue changes in patients with the habits of Smoking (n=5) and Pan chewing (n=5) in comparison with Controls (n=5), by using histo-pathological stains namely,*
   a. *Hematoxylin and Eosin stain*
   b. *Van Gieson stain*
3. *To evaluate and compare these groups, among patients with Leukoplakia (n = 5), Sub mucous fibrosis (n=5), Well-differentiated squamous cell carcinoma (n=5), Moderately differentiated squamous cell carcinoma (n=5) and Verrucous carcinoma (n=5), by using histo-pathological stains namely,*
   a. *Hematoxylin and Eosin stain*
   b. *Van Gieson stain*
4. *To evaluate the changes in fibroblasts in patients (n=5) with habits of smoking (n=2) and pan chewing (n=2) and a control (n=1), by Transmission electron microscopy.*

REVIEW OF LITERATURE

Review of Literature

Most malignancies of the upper aerodigestive tract are squamous cell carcinomas (SCC) arising in the mucous membranes of the mouth, pharynx, and larynx and share common risk factors. Of all oropharyngeal malignancies reported to the SEER registries in the United States between 1973 and 1987, apart from lesions of salivary glands, gingivae, nasopharynx, nasal cavity, and sinuses, more than 95 percent were SCC.[1]

For both genders combined, cancer of the mouth and pharynx ranks sixth overall in the world, behind lung, stomach, breast, colon and rectum, and cervix plus corpus uteri. Cancer of mouth and pharynx is the third most common site among males in developing countries and fourth among females. The highest rates in the world for oral cancer are found in France, the Indian subcontinent, Brazil, and central/eastern Europe [1].

Incidence increases with age in all countries. In the West, 98 percent of oral and pharyngeal cases are in patients over forty years of age. In high-prevalence areas, cases occur prior to the age of thirty-five due to heavy abuse of various forms of tobacco.

Furthermore, a number of cases of oral mucosal SCC occur in both young and old patients often in the absence of traditional alcohol and tobacco risk factors and may pursue a particularly aggressive course.[70]

In industrialized countries, men are affected two to three times as often as women, largely due to their higher indulgence of alcohol and tobacco for intra-oral and laryngeal cancer and higher exposure to sunlight for lip cancer among those who work outdoors. However, the incidence of tongue and other

intra-oral cancer for women can be greater than or equal to that for men in high incidence areas such as India, where chewing and sometimes smoking are also common among women.

Emigrants from high-incidence regions to other parts of the world result in comparatively high rates in immigrant communities. Among Indians in the Malay Peninsular, for example, oral cancer has long been considerably more common than among Malays or Chinese.[71] Similar trends are noted among Indian migrants from India to Natal.[72]

Taken together, the effects of tobacco use, heavy alcohol consumption, and poor diet probably explain over 90 percent of cases of head and neck cancer. The vast majority of individuals who develop squamous cell carcinoma of the upper aerodigestive tract have a history of smoking tobacco[3]. Many of these individuals also consume alcohol regularly[4, 5]. The increased risk for developing head and neck cancer in these individuals has been well established by large epidemiological studies.[4–10]

Among men in industrialized countries, smoking is estimated to be the cause of 40-45 percent of all cancer deaths, 90-95 percent of lung cancer deaths, over 85 percent of oral cancer deaths, 75 percent of chronic obstructive lung disease deaths, and 35 percent of cardio-vascular disease deaths in those aged thity-five to sixty-nine years. Alcohol synergizes with tobacco as a risk factor for all upper aerodigestive tract SCC: this is super-multiplicative for the mouth.[27]

Smoking of tobacco as factory-made cigarettes, cigars and cheroots, and loose tobacco in pipes or hand-made cigarettes is familiar to all. Tar, nicotine,

and nitrosamine content vary greatly, depending on species, curing additives, and method of combustion.

Such smoking habits are the predominant form of tobacco use in the West and among increasing millions in developing countries.

Not only have the smoking of tobacco and the drinking of alcohol each been demonstrated to be independent risk factors for the development of head and neck cancer, but together they have been shown to increase patient cancer risk in a multiplicative manner.[5–7]

The potentiation of the carcinogenic effects of tobacco by alcohol may be a consequence of its solvent properties or its toxicity to the respiratory epithelial enzyme systems.[11, 12] *Alcohol is also able to induce hepatic microsomal enzymes that are capable of transforming proximate carcinogens to ultimate carcinogens.*[11, 12]

Other factors that are believed to contribute to the carcinogenic effect of alcohol include nutritional deficiencies that occur in heavy drinkers and contaminants and congeners that are present in alcohol.[11, 12]*. Unfortunately, smoking and drinking are social behaviors that commonly occur together, with smokers being drinkers and vice versa.5*

Most oral cavity cancers (75%) are located in a horseshoe-shaped area that extends from the anterior floor of the mouth and includes the tonsillar pillar/retromolar trigone complex. It has been suggested that concentrated carcinogens suspended in saliva are pooled in these areas, encouraging carcinogenesis.[13]*. Although the hard palate, buccal mucosa, gingiva, and tongue were once considered common sites for squamous cell carcinoma, studies have*

demonstrated that three specific intraoral areas are most predisposed to develop squamous cell carcinoma in drinkers and smokers [167, 168]. The floor of the mouth, ventrolateral tongue, and soft palate complex (soft palate proper, lingual aspect of the retromolar trigone, and anterior tonsillar pillars) should be regarded as high-risk sites.

The site and size of 222 asymptomatic squamous cell carcinomas were documented in 161 cigarette smokers who were also drinkers. Of 207 intraoral lesions (excluding 15 of the lip), 201 lesions (97 percent) were found in three locations: 101 (50 percent) in the floor of the mouth, 64 (32 percent) in the soft palate complex and 36 (18 percent) in the ventral or lateral tongue.[167]

Examination of the anatomical sites predisposed to manifesting head and neck cancers have demonstrated that the risks of developing oral, pharyngeal, and laryngeal cancers are especially high in individuals who both smoke tobacco and drink alcohol.[7– 10, 12, 14]

Role of Smokeless Tobacco:

Much of the tobacco in the world is consumed without combustion. Rather, it is placed in contact with mucous membranes, through which nicotine is absorbed to provide the pharmacological effect. Use of nasal stuff, popular in the last century, is returning.

Other forms of snuff, loose or in packets and placed in the oral vestibule, are common in Scandinavia and United States.

Tobacco is also prepared in blocks or flakes for chewing. In developing countries, tobacco is mostly consumed mixed with other ingredients. The very extensive evidence for carcinogenicity of these mixtures is covered exhaustively

in Daftary et al[28] and Gupta et al.[29] Toombak, the form used in Sudan, contains very high levels of tobacco specific nitrosamines (TSNs), and users show significantly increased risks of oral squamous cell carcinoma.[30]

Role of Betel Quid:

Quid should be defined as "a substance, or mixture of substances, placed in the mouth or chewed and remaining in contact with the mucosa, usually containing one or both of the two basic ingredients, tobacco or areca nut, in raw or any manufactured or processed form". Thus betel quid is to be considered as a specific variety of quid: it indicates any type of mixture or quid that included betel leaf.

Quid can be categorized into three forms namely,

- *Quid with areca nut but without tobacco products (Areca nut quid)*
    - *Chewing areca nut only*
    - *Chewing betel leaf and areca nut*
- *Quid with tobacco products but without areca nut (Tobacco quid)*
    - *Chewing tobacco*
    - *Chewing tobacco and lime*
    - *Use of mishri (burned tobacco applied to teeth and gums)*
    - *Use of niswar (a type of tobacco snuff)*
    - *Use of naas (a stronger brand of niswar)*
    - *Moist snuff*
    - *Dry snuff*
- *Quid with areca nut as well as tobacco products (Tobacco and Areca nut quid)*

- *Betel quid with tobacco*
- *Betel with tobacco-lime-areca nut mixtures*
- *May contain tobacco leaves (whole or crushed), packaged tobacco products like zarda, gutkha*

Quids are prepared from areca nut, cured or sun-dried, and chopped, then usually placed on a leaf of the Piper betel vine. Slaked lime is an essential ingredient. It lowers pH and accelerates release of alkaloids from both tobacco and nut, with enhanced pharmacological effect.

Daftary et al [31] has reported the evidence for carcinogenicity of betel quid and the important role of tobacco in considerable detail.

Role of Areca Nut Alone:

Although the IARC[32] concluded that there was insufficient evidence that the chewing of betel quid without tobacco was carcinogenic to man, this is a probability. Areca nut is certainly the main etiological agent in oral submucous fibrosis[33] but in this case-control study twelve of the fourteen concurrent oral cancer and submucous fibrosis patients also used tobacco.

In Guam, where areca nut is chewed alone or with leaf only, there is apparently no increase in oral cancer[34.] Conversely in Taiwan, most heavy chewers of betel quid do not include tobacco, yet oral cancer is clearly associated.[35]

As we evaluate the dangers of these complex mixtures, it is important to remember that betel leaf is also protective in nature [36] and at least two compounds have been identified: carotene and hyroxychavicol, an astringent antiseptic.

Brown et al [37] described "snuff-dipper's cancer" in the south-eastern United States due to the habit of placing snuff in the labial sulcus—the basis of the classic description of verrucous carcinoma by Ackermann,[38] confirmed later by McCoy and Waldron.[39]

As Axell [40, 41] points out, oral snuff almost always produces mucosal lesions, often affects salivary flow, causes gingival recession, and creates nicotine dependence and addiction.

Role of Smoking:

The most comprehensive source of evidence for the carcinogenicity of tobacco smoke remains the IARC publication of 1986.[42]

This evidence is summarized by the U.S. Surgeon General's Report of 1989: upper aerodigestive sites have the highest Ars in males, of all the many sites influenced by smoking. A major difficulty in accurately quantifying smoking risks for aerodigestive tract cancer is its strong synergism with alcohol.

Pipe smoking has long been associated with lip cancer, where the nature of the stem and its permeability and, maybe, temperature are cofactors. Some literature suggests that pipe and cigars are less risky for oral cancer than cigarettes [43] but a study from North Italy [27] shows higher risks associated with these practices for cancer of the mouth and esophagus than cigarettes.

The relationship between smoking and the anatomical site of oral cancer is less clear-cut than with smokeless tobacco. Pooling of carcinogens in saliva leads to cancers in the "gutter" area—floor of the mouth and ventral and lateral tongue.

Mashberg and Meyers [44] reported in a U.S. population that 201 of 207 asymptomatic, primarily erythroplastic carcinomas were in three locations: floor of mouth (101 carcinomas), ventral or lateral tongue (36 carcinomas), and soft palate (64 carcinomas).

In the Amsterdam series [45] the floor of mouth and retromolar area were significantly more related to tobacco use than cancers or the tongue and cheek.

However, in another series of 359 male cases among U.S. veterans, smoking was more strongly associated with soft palate cancers than anterior sites, and alcohol was associated with floor of mouth lesions.[46] This is interesting because the long-recognized lesions of stomatitis nicotina ("smokers palate") have a low malignant potential (except in reverse smokers). Stomatitis nicotina, in the West, is most commonly associated with pipe smoking and both hard and soft palate is relatively uncommon sites of oral cancer.

Bidis contain much higher levels of phenol, hydrogen cyanide, and benzo(a)pyrene than conventional cigarettes [61]. Bidis and kreteks may in fact produce carcinogens other than those commonly found in tobacco. Flavoring agents added to bidis are not rigidly controlled, so the composition may be variable. Mutagenicity testing of flavoring agents is commonly done using aqueous or organic extracts. However, testing of the parent compound in this way may not identify potentially hazardous pyrolysis products formed during combustion of the flavoring agent. Combustion of virtually all organic matter produces polycyclic aromatic hydrocarbons, some of which may be metabolized to form carcinogenic epoxides.

Clove cigarettes contain the genotoxic phenyl propenes, safrole, eugenol and methyl eugenol which are established or suspected carcinogens in animal models [62]. Furthermore, some spices that are not mutagenic based on an Ames assay of conventional extracts become mutagenic when treated first with nitrite [63]. Nitrite is abundant in tobacco, where it is the primary nitrosating agent [64].

Adolescents and young adults may be particularly susceptible to exposures to oral carcinogens. Epidemiological studies [65] and mathematical carcinogenesis models [66] implicate young age at initiation of smoking as an independent risk factor for cancer. A recent study also showed that, at least in former smokers, young age was associated with higher levels of DNA adducts [67].

It is possible that the oral cavity may also show age-related susceptibility. Before puberty, children are not very susceptible to inflammatory conditions of the oral cavity such as gingivitis and periodontitis. However, puberty brings changes in oral mucosal cell proliferation rates, bacterial populations, and hormonal stimulation [68, 69]. All of these can potentially contribute to altered sensitivity to carcinogens.

The Mechanisms of Tobacco Carcinogenesis:

More than 300 carcinogens have been identified in tobacco smoke or in its water-soluble components that would leach into saliva. [42] The major and most studied of these are the aromatic hydrocarbons - benz-pyrene and the tobacco specific nitrosamines (TSNs), nitroso-nor-nicotine (NNN), nitroso-pyrrollidine (NPYR), nitroso-dimethylamine (NDMA), and 4-

(methylnitrosamino)-1- (3-pyridyl) - 1-butanone (NNK). Benz-pyrene is a powerful carcinogen, with twenty to forty nanograms per cigarette.[47]

Hoffman and Hecht [48] *reviewed the role of N-nitrosamines. Mainstream cigarette smoke can contain NNN and NNK. These are generated primarily during pyrolysis, but also endogenously from some smokeless tobacco. They act locally, on keratinocyte stem cells, and are absorbed and act in many other tissues in the body. They produce DNA adducts, principally methyl Guanine, which interfere with DNA replication. There is damage to all replicating cells, including those of the immune response. Metabolism of these carcinogens usually involves oxygenation by p450 enzymes in cytochromes, and then conjugation, in which the enzyme glutathione S transferase (GST) is involved. Polymorphisms of the p450 and GST genes are currently under active study in the search for genetic markers of susceptibility to head and neck cancer, and indeed to tobacco-related cancers at many other body sites.* [49]

Role of Alcohol:

Pure ethanol has never been shown to be carcinogenic in vitro or in animal studies.[42] *It is presumed to act in concert with other, more direct, carcinogens in the beverage—so-called congeners—and with other environmental carcinogens, especially from tobacco.*

Nevertheless, an increased risk of upper aerodigestive tract cancer associated with alcohol drinking in non-smokers has been demonstrated. [50] *The increase in oral cancer in the Western world has been related to rising alcohol use. In England and Wales, alcohol consumption per capita fell from the turn of the century to the 1930s, but has more than doubled since.*

Using mortality from liver cirrhosis as a surrogate measure of damage to health from alcohol, Hindle [51] has plotted trends over this century and shown how they closely match the trends in oral cancer mortality. Taking deaths from lung cancer as a measure of tobacco damage, it is striking how the trends for oral cancer move, both down and up this century, in opposite directions: strong circumstantial evidence that alcohol rather than tobacco is the major factor in the observed trends in oral cancer mortality and, by inference, incidence.

All forms of alcoholic drinks are dangerous if heavily consumed, the most dangerous reflecting the predominant habit in the population under study. Thus there is evidence for the role of beer[52, 53] wine [54, 27] and spirits [55].

It is notable that the 95 percent confidence intervals for the odds ratio for all beverages do not cross unity until the total consumption is really quite high - above fifty-five drinks per week. This is consistent with other data.[56]

When the tobacco effect is adjusted for, heavy alcohol consumption itself produces considerable risks, with ORs or RRs of 17, 23, 33, and 70 appearing for oral cancer in the different studies. For these high rates of alcohol use/abuse, the risks are greater than for tobacco, adjusted for alcohol. Self-reported alcohol consumption tends to be underestimated, implying that alcohol may be even more important.

Alcohol is also high in calories, which suppresses appetite. Those with a serious drinking problem become socially fractured, and many choose to spend available cash on drink rather than food. All of this contributes to inadequate diet. Metabolism is further damaged by liver disease. As a result, nutritional deficiencies are common.[57]

Effects of alcohol on oral mucosa:

There was a highly significant decrease in the mean cytoplasmic area and a significant reduction in the mean nuclear area of cells from the alcohol group which did not vary with age. This may be due to the direct effect of alcohol, which may have a dehydration effect.

Mascres et al [148] observed epithelial atrophy in esophageal mucosa of rats after chronic alcohol ingestion, which they associated with a reduction in basal cell size.

Maier et al [149] observed epithelial atrophy on oral mucosa of rats after chronic alcohol consumption. However, they found an increase in basal cell size which they attributed to hyper-regeneration in response to ethanol cytotoxicity. They attributed the epithelial atrophy to a reduction in thickness of the maturation cell layer. However, they did not measure cell size in this layer, so it is unclear if this was due to a reduction in the number of cell layers or to a reduction in the size of the cells in the maturation layer.

Valentine et al [150] noted epithelial atrophy in human lingual epithelium due to alcohol and tobacco consumption. He found an increase in basal cell size associated with alcohol and a decrease in size of the more superficial cells.

Anderson [151] looked at oral smears from 3445 dental patients and 276 alcoholic patients over a three year period. He found a statistically significant increase in the number of dyskeratotic cells within the alcohol group compared with that of controls.

Hillman and Kissin [152] looked at oral cytology patterns in relation to smoking within 790 alcoholic subjects. They found a significant increase in nuclear and cell size in smokers compared with non-smokers.

Ogden et al [153] assessed the effects of tobacco smoking of more than twenty cigarettes per day, in non-anemic, age and gender matched controls and found out that there was only a slight increase in nuclear level (5%).

Mechanism of Alcohol carcinogenesis:

Most alcohol is metabolized by alcohol dehydrogenase into acetaldehyde, a highly toxic substance suspected to cause the tissue damage attributed to alcohol ingestion. However acetaldehyde is rapidly metabolized to a non-toxic form of acetate by aldehyde dehydrogenase. Thus individual variation in the oxidation of ethanol to acetaldehyde and in particular, the length of time required to catalyze the conversion of acetaldehyde to acetic acid by acetaldehyde dehydrogenase (ALDH).

Alcohol dehydrogenase and Aldehyde dehydrogenase activity have been demonstrated in the oral cavity [154]. Individual variations in such enzyme levels may help explain different oral cellular responses between individuals within the alcohol group.

There is strong epidemiological evidence that combined use of alcohol and tobacco has a synergistic effect in the etiology of oral cancer. The concentration of tobacco-specific nitrosamines is very high in saliva and that extended contact with oral mucosa occurs with usage of smokeless tobacco. Ethanol has been shown to be a penetration enhancer for epithelial tissue [155] and there could be increased penetration of the carcinogens into the oral epithelium.

Ethanol is used as an effective permeability enhancer for drug delivery across skin, with a maximum effect at concentrations between 20 – 40%, depending on the nature of the penetrating compound [156]. This effect may reflect enhancement of a pathway through a liquid crystal lipid phase of the intercellular lipid barrier of the stratum corneum [157].

A similar mechanism may explain permeability enhancement in non-keratinized oral mucosa where the epithelial barrier is also represented by intercellular lipids, although probably with more extensive liquid crystalline membrane domains and less extensive gel phase domains than in epidermis [158]. The absence of significant permeability effect with 50% ethanol could be due to the fixative effect of higher concentrations of ethanol in tissue, perhaps involving dehydration of the more aqueous pathways penetrating the liquid domains of the intercellular barrier [156]. Studies reveal that 25-30% ethanol significantly increased the penetration of NNN across the floor of mouth mucosa. Since the floor of the mouth is the most permeable area of the oral cavity, and since this region has been suggested to have the highest risk for developing oral cancer, the permeabilizing effect of alcohol could explain the synergistic role of alcohol with tobacco [159]. The risk of cancer with smoking and drinking is tabulated below,

Head and Neck Cancer in Non-Users of Tobacco and/or Alcohol:

A minority of patients develop a cancer in the apparent absence of one or both of these risk factors.

Rich and Radden [58], the Amsterdam data [45], Hodge et al [59] , Bross and Coombs[15], Wiseman et al[2], Koch WM, Lango M, Sewell D, et al[16], Blot et al[5],

Llewellyn et al [19], Constantinides et al.[20], Agudelo D et al [21], Lemon FR, Walden RT, Woods RW.[22], Phillips RL[23], de Boer MF, Sanderson RJ, Damhuis RA, et al [24], Hodge et al [17], Panis et al [18], Kotwall et al [25], Slaughter et al [26] have reported the incidence of oral cancer in patients who are non-users of tobacco and alcohol.

None of these studies on the rare cases that occur in non-users of tobacco and/or alcohol dilutes the evidence that these are far and away the major risk factors. Viral infections and nutritional inadequacies are the main hypothetical factors in this group of patients.

Verrucous carcinoma:

The first case report was published in 1941 by Friedell and Rosenthal [166] who described it as papillary verrucoid carcinoma. Then in 1948, Lauren V. Ackerman [160, 167] reported a series of 31 similar oral cases and described a neoplasm of the oral mucous membrane, which he thought represented a unique type of squamous cell carcinoma and coined the term verrucous carcinoma with the characteristic clinical and histological findings.

Verrucous carcinomata are rare tumors of the oral cavity, representing anywhere from 1 to 10% of all oral squamous malignancies [163]. The most common sites of involvement in the mouth are the buccal mucosa and gingiva, the alveolar ridge, the palate, and the tongue. Compared with conventional squamous cell tumors of the head and neck, they tend to present at an advanced age, with a higher proportion of female patients [164].

Although these tumors are classified as carcinomata, they are extremely well-differentiated rare variants of squamous carcinoma with little or no

metastatic potential. However, they cause significant morbidity because of their local invasiveness and their pattern of stubborn recurrence with the currently acceptable modalities of radiation and surgery [165].

The formal definition [167] refers to a warty and densely keratinized surface. The lesion is contained within a sharply circumscribed deep margin. Close inspection reveals bulbous well-oriented rete ridges with well-keratinized squamous epithelium with no anaplasia. One of the most distinguishing features is a pushing rather than infiltrating type of advancing margin. There is associated inflammation in the adjacent stroma.

Prioleau et al [161] performed a study on verrucous carcinomas of the rectum, plantar surface of the foot, and oral cavity were studied by means of light and electron microscopy, and autoradiographic and immunofluorescent techniques. Histologic examination showed that each tumor was composed mainly of mature squamous epithelium, and each had foci of slight cellular atypia. The cells in S-phase consistently were situated near the basal layer. Immunofluorescent examination with antibasement membrane antibody showed areas of marked focal thickening and other areas where basement membrane was absent. Ultrastructural examination showed reduplicated as well as normal basal lamina. Numerous interdigitating microvilli and well developed desmosomes characterized the cells above the basal layer. A proliferative basal zone underlying a thick layer of well differentiated non-proliferating keratinocytes and reduplicated basal lamina were seen in all tumors, regardless of location. These consistent findings constitute evidence that

verrucous carcinoma is a morphologic and cytokinetic entity that may occur in multiple anatomic sites.

Jiang et al [162] studied the immunohistochemical alteration of basement membrane (BM) type IV collagen and laminin in oral verrucous carcinoma and its BM ultrastructural variations in 16 cases of oral verrucous carcinoma (OVC), 10 cases of oral squamous cell carcinoma (OSCC) and 9 cases of oral mild to severe epithelial dysplasia (OMSD) by using a immunohistochemical S-P method, and the results were analyzed by quantitative method. 3 cases of OVC were observed by TEM. The BM in OVC was thicker than in OSCC and OMSD. TEM found the basal lamina in some areas showed a marked reduplication. The BM in OVC was generally intact (13/16), whereas in OSCC it was mostly discontinuous (9/10), especially around the neoplasm front or the small cord consisted of a few cells, and mostly continuous in OMSD (6/9). There was a stromal inflammatory infiltration around tumor nests for all the oral lesions, but it was much heavier in OVC than that in OSCC and OMSD ($P < 0.05$). There was a positive correlation between intraepithelial lymphocytic infiltration and the BM continuity for OVC ($P < 0.01$). He concluded that the more continuous BM and the heavier inflammatory infiltration in the connective tissue of OVC may be related to its biological behavior.

Leukoplakia:

The term leukoplakia was first used by Schwimmer in 1877 to describe a white lesion of the tongue, which probably represented a syphilitic glossitis. [170]

In 1978, World Health Organization group defined oral leukoplakia as, "A white patch or plaque that cannot be characterized, clinically or histopathologically, as any other disease"[146].

At the international seminar held in 1983, the outcome of which was published in 1984 [80]*, it was decided to avoid the use of the term leukoplakia where there was a known etiological factor, except in those cases where tobacco was believed to be the cause.*

Thus the previous definition was revised to "Leukoplakia is a whitish patch or plaque that cannot be characterized clinically or pathologically as any other disease and it is not associated with any physical or chemical causative agent except the use of tobacco".

The etiological description identified two categories of leukoplakia: those of unknown etiology (idiopathic) and those associated with, or thought to result from, the use of tobacco (tobacco-associated).

The clinical variants to be recognized are,

1) Homogeneous leukoplakia:

2) ***Non-homogeneous leukoplakia:***

Proposal for a LCP Classification and Staging system for Oral leukoplakia:

L prov (isional): *A provisional diagnosis of oral leukoplakia is made when a lesion at clinical examination cannot be clearly diagnosed as any other disease of the oral mucosa with a white appearance.*

L def (initive): *A definitive diagnosis of oral leukoplakia is made as a result of the identification, and if possible, elimination, of suspected etiologic factors and, in the case of persistent lesions, histopathological examination.*

L prov:

1st symbol (L) represents the size:

1 = < 2cm 2 = > 2 to 4 cm 3 = > 4cm x = Not specified

2nd symbol (C) represents the clinical aspect,

1 = homogeneous 2 = non-homogeneous x = Not specified

L def:

3rd symbol (P) represents the pathological features:

1 = no dysplasia 2 = mild dysplasia 3 = moderate dysplasia

4 = severe dysplasia x = not specified

Stage grouping: *(only for leukoplakias that have been examined histologically)*

Stage 1: any L C1 P1 P2, Stage 2: any L C2 P1 P2, Stage 3: any L any C P3 P4

Prevalence of Oral Leukoplakia:

According to well-documented epidemiologic data from different countries over the last thirty years, the prevalence of oral leukoplakia varies between 1.1 and 11.7 percent, with a mean value of 2.9 percent. [73 - 79]

This range reflects assessments made on the basis of different definitions of oral leukoplakia, [80 -82] *which can result in different prevalence rates.*

Smoking and the Prevalence of Oral Leukoplakia:

Early descriptive studies (performed mainly in India and Denmark) have shown that the frequency of oral leukoplakia among smokers is so high that, in the absence of controls, the habit could be considered as causative.

Cross-sectional studies indicate the risk for oral leukoplakia between smokers and nonsmokers. The smoking habits in India show a varying

association with locally prevailing tobacco habits, i.e., chewing, smoking, and mixed habits (chewing betel quid and bidi smoking). All habits were associated with the onset of oral leukoplakia, and the prevalence was considerably higher among the tobacco-using groups than the non-users.

Renstrup [83], Pindborg, Roed-Petersen, and Renstrup [84],Roed-Petersen and Pindborg [85], Mehta et al. [86], Dombi et al.,[87], Bruszt[88] have reported the association of smoking and incidence of leukoplakia.

The studies that report on the distribution of leukoplakia cases according to smoking habits are Banoczy and Rigo [89], a case-control study from Kenya [90], Downer's study [91] and Winn [79].

Evidence from Tobacco Intervention Studies:

A decrease in the prevalence of oral leukoplakia after smoking cessation has been observed in many studies, confirming an etiological role.

Banoczy [92], Roed- Petersenís study [93], Gupta et al [94] have reported resolution of leukoplakia after cessation of smoking habits.

In a recent study of 3,051 male U.S. military trainees, among the 302 individuals using smokeless tobacco, 39.3 percent had leukoplakia compared to 1.5 percent among non-users. After six weeks of tobacco cessation, 97.5 percent of leukoplakic lesions showed complete resolution clinically. [95]

Role of Smoking in Malignant Transformation of Oral Leukoplakia:

The studies cited above mostly provide evidence of the role of smoking in the development of oral leukoplakia. However, there is also strong evidence of a relationship between tobacco use and the development of oral cancer, although

it is not clear whether smoking promotes the development of cancer from oral leukoplakia.

Earlier studies have showed an increased risk of malignant transformation of oral leukoplakia among non-smokers. Einhorn and Wersall [96] cite an eight-fold risk in Sweden, and Roed-Petersen [93] a fivefold risk in Denmark.

Banoczy's [97, 98,] Silverman, Rozen [99], Silverman, Gorsky, and Lozada [100] have reported varied rates of transformation of leukoplakia into carcinoma.

However, as relatively few leukoplakias transform into cancer, it is difficult to determine the role of tobacco in this process.

Histopathology of Leukoplakia:

Loning T and Burkhardt A(1979) [171] assessed the sub-epithelial and peritumoral inflammatory infiltrates of 202 oral premalignant and malignant lesions -- 108 leukoplakias and 94 squamous cell carcinomata with different grades of dysplasia using an immuno-enzymatic method. The incidence of immunoglobulin labeled plasma cells (IgA and IgG) was twice as high in those cases of leukoplakia where dysplasia was present. The number of plasma cells, especially IgA- and IgG-containing plasma cells, decreased significantly with progressive tumor dedifferentiation. In the epithelium, IgA and IgG were localized throughout all epithelial layers in leukoplakias with dysplasia. This finding indicates a leakage of locally synthesized immunoglobulins through an altered oral mucosa. This investigation revealed alterations in the local immune homoeostasis of the oral mucosa in premalignant and malignant lesions which varies with the grade of dysplasia, tumor differentiation and therapy.

Rodriguez-Perez I and Banoczy J (1982) [172] analyzed the histological sections from 100 leukoplakia patients in order to define the characteristics of epithelial and connective tissue alterations and the possible correlation of these changes. Parakeratosis was found in 39% of the cases, orthokeratosis in 24% and both types of keratinization in 34% of the cases. The most characteristic alteration of the thickness of the epithelium was hyperplasia in 81% of the cases. Epithelial dysplasia was present in 27% of the samples. A chronic inflammatory infiltration was generally present in the subepithelial region and in some cases within the epithelium. An increase in the number of these cells related to hyperplasia, parakeratosis and dysplasia has also been found. These results may be interpreted as a possible relation between epithelial and connective tissue changes in leukoplakia lesions.

Banoczy J, Lapis K, Albrecht M (1980) [173] studied the biopsy specimens of buccal mucosa from six oral leukoplakia patients and one specimen of normal buccal mucosa by scanning electron microscopy. The results showed a difference between the surfaces of normal and leukoplakic epithelial cells. The appearance varied with the clinical type of leukoplakia: in leukoplakia verrucosa, epithelial cells appeared keratinized; in leukoplakia erosiva, epithelial cells were dissociated. Histologically, dysplastic erosive leukoplakia was characterized by an atypical arrangement of superficial cytoplasmic projections of epithelial cells.

Bondad-Palmario GG (1995) [174] analyzed the phenotype and distribution of immunocompetent cells in 100 cases of oral leukoplakia with different levels of dysplasia. Cells were identified in two compartments of the oral mucosa, the

epithelium and subepithelial connective tissue. In the main lymphoid population of each groups, the T lymphocytes predominated over the B lymphocytes. The lymphoid cells were present either as diffuse aggregates or organized in follicular patterns with or without germinal center-like structures. When present, B lymphocytes were seen to constitute the above mentioned structures. T lymphocytes made up the paracortical areas. A decrease in CD4/CD8 ratio was observed in cases with severe dysplasia. Specimens classified as mild to severe dysplasia presented a significant increase in the number of CD1a (+) dendritic Langerhans cells when compared with those of epithelial hyperplasia. A significant increase in macrophage count was also obtained in the sub-epithelial connective tissue of all dysplastic cases. A significant increase of CD57 (+) natural killer/killer cells in the subepithelial connective tissue and HLA-DR expression by the keratinocytes was observed in cases with severe dysplasia. Correlation and analysis of the results revealed an immuno-cellular reaction that varied according to the degree of dysplasia in oral leukoplakia. Immunologic events, i.e. decreased CD4/CD8 ratio, increased density of natural killer/killer cells and HLA-DR expression by keratinocytes, occurring simultaneously in severe dysplasia are speculated to be indicative of early malignant transformation.

Effect of Smoking on Clinically Healthy Oral Mucosa:

Several investigators have studied the question of whether the structure of the clinically healthy oral mucosa shows any alterations in smokers.

Banoczy [101] reported on the results of cytological examination of oral smears in 100 healthy, male and female smokers and non-smokers. Evaluation

of the keratinization pattern revealed a significant increase in keratinized cells in the epithelium of the tongue and hard palate of both male and female smokers, when compared with non-smokers.

Meyer, Rubinstein, and Medak [102] took smears of ten clinically normal regions from ninety-nine subjects and found that smoking affected keratinocytes differently in different regions, depending on the extent of direct exposure to smoke. The initial changes were more marked in non-keratinized than in keratinized regions and, interestingly, were in the direction of a less differentiated cell type. The results of both studies point to cellular alterations preceding the clinical changes.

Oral Sub mucous fibrosis:

It is approximately half a century since Schwartz described this condition in the tobacco-chewing women of Indian origin in Kenya. Since then this condition evoked an intense enthusiasm among many researches in India and through out the world.

Various authors had investigated the condition thoroughly and proposed several factors that play a role in the etiopathogenesis of this condition. Current evidence suggests that arecoline in the areca nut is the key factor in initiating the disease process.

This condition is aptly described by Pindborg and Sirsat as "an insidious chronic disease affecting any part of the oral cavity and some times the pharynx. Although occasionally preceded by and/or associated with vesicle formation, it is always associated with a juxta-epithelial inflammatory reaction followed by a

fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat".

The habit of betel quid chewing is widespread throughout India and South East Asia. This condition is also reported in Asian immigrants living in other parts of the world.

Various researchers have conducted studies in different parts of the country to check out the incidence of betel quid chewing habit in general population. This habit is widely prevalent in teenagers and young adults.

Oral submucous fibrosis predominantly involves the oral cavity. The buccal mucosa, retromolar area, and the soft palate are the predominantly affected sites. The mucosa in the involved areas gradually becomes pale followed by progressive stiffness of subepithelial tissues.

In addition to the involvement of oral mucosa, this condition also involves the pharynx and esophagus in persons who chew and swallow the products of betel quid.

Recently, an increased incidence of malignancy is noted in oral submucous fibrosis patients, particularly in people who use commercially available products. The malignant transformation of betel quid users in India is around 8%, which is quite high [115, 145].

The most ironical aspect of this condition is lack of appropriate treatment modalities. Unlike tobacco pouch keratosis, oral submucous fibrosis does not regress with the habit cessation, although mild cases may be treated with intralesional corticosteroids to reduce the symptoms.

Surgical splinting and excision of the fibrous bands have also been tried to improve the mouth opening in later stages of the disease. A recent study showed that intralesional injections of interferon gamma improved maximum mouth opening, reduced mucosal burning and increased suppleness of the buccal mucosa.

Historical Review:

This condition was first described in ancient Indian Medical Manuscripts by Sushruta at the time of around 400 B.C. describing it as "VEDARI", where he gives description of patients suffering from narrowing of mouth, burning sensation and pain.

Schwartz (1952) reported a condition consisting of limitation of mouth opening amongst south Indian women in Kenya, which he named "atrophia idiopathica (tropica) mucosa oris".

Joshi (1953) an ENT surgeon observed this condition and termed as "submucous fibrosis of palate and pillars of fauces".

Of all the terminologies in the literature, the term "Oral Sub-mucous Fibrosis" is currently widely used.

Etiology:

Although various etiological agents are proposed, the exact etiology of oral submucous fibrosis has not yet been identified. Current evidence suggests that arecoline in betel nut plays a major role in initiating the disease process.

Various etiological agents are summarized by Abrol.

Su (1954), Rao (1962), Sirsat & Khanolkar (1962), Gupta D.S. et al (1980), Pindborg & Sirsat (1964), Abrol & Krishnamoorthy (1970), Abrol & Raveendran

(1972), Ramanathan K (1981), Canif. J.P & Harvey.W (1986)[117], Sinor P.N. et al (1990) [118], Binnie & Cawson (1972) [119], Caniff & Pillai, Gollinick et al, Khanna. J. N & Andrade. N. N (1995) [120] have reported the various etiological factors for sub mucous fibrosis..

Malignant potential:

Paymaster first reported the development of slow growing oral carcinoma in 1/3rd of cases seen at Tata Memorial Hospital, Bombay (J.J. Pindborg et al, 1966).

Pindborg et al (1975) [121] , Caniff. J.P et al (1986) [122], Pindborg & Murthy, Shiau & Kwan, Glenn Muraw et al (1987), Maher. R. et al (1996) [123] have reported varied incidences of dysplasia and malignant transformation in sub mucous fibrosis.

Clinical features:

Age incidences given by various authors, based on their studies varied between 10 to 60 years.

Sex incidence also varies amongst various studies, most authors suggested a male preponderance, but Maher (1996) [123] have given an increased female predilection.

The sites commonly involved according to Wahi et al (1966) [124], are palate (51.3%), buccal Mucosa (44.2%), tongue (2.7%), lip and gingiva (0.9%).

Wahi et al (1966)[124] classified submucous fibrosis into three clinical groups namely, Group I, Group II and Group III.

Pindborg et al (1966) [125], McGurk et al Gupta et al (1980) [115], Caniff & El-Labban (1985) [126], Vaish et al (1981) [127], Glenn Morawetz et al (1987), Caniff. J.P

et al (1986)[122], Moos and Madan 1968) [130,] Wahi et al 1966) [124], Pindborg et al 1980) [131] have reported variations in clinical features of sub mucous fibrosis.

Borle. R.M & Borle. S.M (1991) [129] have classified oral submucous fibrosis clinically into two phases, an eruptive phase and an fibrosis induction phase.

Histo-pathological features:

Joshi (1953) mentions frequent presence of intra-epithelial vesicles in early stages of disease. Other changes include parakeratosis, signet cell degeneration, liquefaction degeneration of basal layers.

Pindborg J.J, Mehta F.S, Daftary D.K [121] reported that out of 53 cases of biopsy specimens observed, 71.7% of biopsies showed atrophic epithelium, normal thickness in 26.4% and hyperplastic epithelium in 1.9%. In 26% of cases, buccal mucosa showed hyper-orthokeratosis, 22% showed hyper-parakeratosis and 52% showed non-keratinized surface. 22.6% cases showed epithelial atypia with intercellular edema. 19.2% of biopsies showed signet cells in basal layer. There was reduction of melanin pigment in basal cell layer and 3 biopsies revealed presence of colloid bodies in epithelium and marked lymphocyte infiltration in lamina propria.

McGurk et al (1984) [132] observed subepithelial chronic inflammatory reaction and accumulation of dense collagen at dermo-epidermal junction with extension of the fibrosis down into the submucous and voluntary muscle.

El-Laban NG and Caniff J.P. (1985) [133] studied ultra structural findings of muscles degeneration in OSMF. He demonstrated severe necrosis in high proportion of muscle fibers.

Caniff J.P, Harvey, Harris (1986) [122] examined 30 cases and showed atrophic epithelium in 26%, 33% had non-keratinized epithelia, 27% had mild and 7% showed moderate atypia. All 30 cases (100%) showed collagen accumulation beneath basement membrane and chronic inflammatory cell infiltrate consisting of lymphocytes, plasma cells monocytes, and macrophages within lamina propria.

De Waal et al (1997) [134] studied the fibroblast content in SMF. They observed an increase in F-3 cells which produced type I and type III collagen in excess amounts in oral submucous fibrosis.

Immunological, Biochemical and Hematologic Features:

Pindborg et al, Dinesh. S. Gupta et al (1980) [135] , Rajendran R et al (1986) [136], Canif J.P, Harvey, Harris (1986) [122,] Glenn Morawetz et al (1987) [128], Chaturvedi V. N & Marathe. N. G. (1988) [138,] Anuradha. C. D and Shyamala Devi C. S (1998) [139] have reported the immunological and hematological variations in sub mucous fibrosis.

Scutt A et al (1987) [137] observed that treatment of reconstituted collagen fibrils and pieces of rat dermis with the crude extract, purified tannins or (+)-catechin from betel nut (Areca catechu) increases their resistance to both human and bacterial collagenases in a concentration-dependent manner. These tanning agents may stabilize collagen in vivo following damage to the oral epithelium, and promote the sub-epithelial fibrosis which occurs in betel nut chewers.

Haque. M.F et al (1997) [140,] Kaur J et al (1999) [141,] Trivedy C et al (1999) [142,]

Chiang CP et al (2000) [143], Mythily Srinivasan et al (2001) [144] have evaluated the various histochemical and molecular changes in sub mucous fibrosis.

Mollenhauer and Bayreuther [208] described three distinct fibroblast cell forms in rat connective tissue that can be identified on the basis of their morphology. They can also be distinguished from one another by the amount and type of collagen synthesized.

1. *The F1 fibroblast is spindle shaped, highly proliferative and secretes low levels of type I and III collagen.*
2. *The F2 fibroblast is more epitheloid, less proliferative and synthesizes relatively more collagen.*
3. *The F3 fibroblast is a large stellate cell and the least proliferative, produces four to eight times more types I and III collagen than F1.*

According to these workers, F2 cells sequentially arise from F1 cells and F3 cells sequentially arise from F2 cells.

Role of sub-epithelial connective tissue and inflammatory cells in differentiation of epithelium:

Squier CA and Kammeyer GA (1983) [175] performed a study in which the specimens of buccal mucosa and ear skin were introduced into the uteri of isologous rats and the animals maintained under estrogen stimulation for ten weeks, whereupon the uteri were removed and processed for light- and electron-microscopic examination. The majority of implants were successful with the epithelium having migrated to replace the adjacent uterine epithelium. Epidermis and oral epithelium growing on its own connective tissue in the uterus showed a normal pattern of histodifferentiation, including the formation

of a thickened keratinized surface and appendages such as hair and sebaceous glands. Ultrastructurally, maturation was similar to that of normal tissue. Epidermis growing on uterine connective tissue did not form appendages but the ultrastructural pattern of cytodifferentiation was similar to control tissue. Buccal epithelium on the uterine stroma was markedly altered in its pattern of maturation, the epithelium appearing thinner than usual and showing a pattern of maturation resembling that of non-keratinization. These results suggest that normal histodifferentiation in epidermis and oral epithelium requires the presence of the appropriate connective tissue; in its absence epidermis has an intrinsic capacity for more or less normal cytodifferentiation but this is lacking in the buccal epithelium.

Mackenzie IC, Hill MW (1984) [176] assessed the epithelial-mesenchymal interactions and their influence on the maintenance of epithelial structure in the adult. To examine this problem, separated epithelial and connective tissue components of skin and mucosae from various regions of adult mice were homo-or heterotypically recombined and transplanted to histo-compatible hosts. The patterns of tissue architecture and keratinization of the resultant epithelia were examined for changes indicative of mesenchymal influences on the epithelial phenotype. Each type of epithelium, in some recombinations, fully conserved its normal pattern of phenotypic expression indicating that subepithelial connective tissue from all regions is permissive and that regionally-specific connective tissue influences are not necessary for conservation of epithelial specificity. In other recombinations, however, the epithelium acquired features of tissue architecture or keratinization typical of

the epithelium normally associated with the connective tissue component, indicating directive influences from the connective tissue. The patterns of epithelial response observed suggest that there may be separate connective tissue influences on epithelial architecture and cyto-differentiation and that there is a regionally-related variation in the competence of epithelia to respond to these influences.

Hill MW, Mackenzie IC (1989) [177] assessed the role of subepithelial connective tissue in modulating the pattern of histodifferentiation and epithelial proliferative activity of stratified epithelia from adult animals. Epithelial and connective tissues of murine skin and oral mucosa, differing in their morphology and proliferative activity, were separated and heterotypically recombined prior to grafting to histocompatible hosts. After 3 or 8 weeks in situ, mitotic activity was determined following the administration of vinblastine sulfate. Although the mitotic activity in each of the epithelia could be modulated by some connective tissues, there was no distinct pattern of behavior. In combination with connective tissues from tongue or palate, the ear epidermis acquired a significantly increased mitotic activity. In contrast, when oral epithelia with high mitotic activity were recombined with dermal connective tissue, there was usually a significant reduction in proliferative activity. As there was no apparent association between mitotic activity and the induced changes in either organization or histodifferentiation, it is suggested that subepithelial connective tissue is capable of directly influencing the mitotic activity in the overlying epithelium.

The stratified squamous epithelia differ regionally in their patterns of morphogenesis and differentiation. Although some reports suggested that the adult epithelial phenotype is an intrinsic property of the epithelium, there is increasing evidence that subepithelial connective tissue can modify the phenotypic expression of the epithelium. Okazaki et al [178] elucidated whether the differentiation of cutaneous and oral epithelia is influenced by underlying mesenchymal tissues. Three normal skin samples and three normal buccal mucosa samples were used for the experiments. Skin equivalents were constructed in four ways, depending on the combinations of keratinocytes (cutaneous or mucosal keratinocytes) and fibroblasts (dermal or mucosal fibroblasts), and the effects of subepithelial fibroblasts on the differentiation of oral and cutaneous keratinocytes were studied with histological examinations and immunohistochemical analyses with anti-cytokeratin (keratins 10 and 13) antibodies. For each experiment, three paired skin equivalents were constructed by using single parent keratinocyte and fibroblast sources for each group; consequently, nine (3 x 3) organotypic cultures per group were constructed and studied. The oral and cutaneous epithelial cells maintained their intrinsic keratin expression. The keratin expression patterns in oral and cutaneous epithelia of skin equivalents were generally similar to their original patterns but were partly modified exogenously by the topologically different fibroblasts. The mucosal keratinocytes were more differentiated and expressed keratin 10 when cocultured with dermal fibroblasts, and the expression patterns of keratin 13 in cutaneous keratinocytes co-cultured with mucosal fibroblasts were different from those in keratinocytes co-cultured with cutaneous fibroblasts. The

results suggested that the epithelial phenotype and keratin expression could be extrinsically modified by mesenchymal fibroblasts.

In an effort to come to a better understanding of human oral mucosal carcinogenesis, Rich AM and Reade PC (2001) [179] used an animal model in which the carcinogen - 4-nitroquinoline-1-oxide was applied to rat palatal mucosa for varying periods of time. Histological and histometric analyses showed that there were quantifiable differences in the palatal epithelium to which carcinogen had been applied in comparison with control tissue. Tissue recombination experiments, using various combinations of the palatal mucosa and analyzed after recovery from transplantation to hypothymic BALB/c mice, showed that control epithelium recombined with connective tissue from carcinogen-treated mucosa was altered, indicating that the underlying connective tissue modified histomorphological aspects of the epithelium in the later stages of carcinogenesis

Gannot et al (2002) [180] assessed the mononuclear infiltration in epithelium derived lesions of various pathological manifestations by histology and immunohistochemistry. The infiltrate under the transformed epithelium of oral lesions, was examined for differences in the composition of immune mononuclear cells as the epithelium moves from hyperkeratosis through various degrees of dysplasia to squamous cell carcinoma. The study was performed on 53 human tongue tissues diagnosed as hyperkeratosis (11 cases), mild dysplasia (nine cases), moderate and severe dysplasia (14 cases) and squamous cell carcinoma (19 cases). Immunohistochemical analysis of various surface markers of the tumour infiltrating immune cells was performed and

correlated with the transformation level as defined by morphology and the expression of p53 in the epithelium. The results revealed that, in the tongue lesions, the changes in the epithelium from normal appearance to transformed were accompanied by a corresponding increase in the infiltration of CD4, CD8, CD14, CD19+20, and HLA/DR positive cells. The most significant change was an increase in B lymphocytes in tongue lesions, that was in accordance with the transformation level (P<0.001).

Autofluorescence and History of Fluorescent Probes:

Emission of light by matter (luminescence) has always been known to man. Lightning in the sky, light emission by bacteria in the sea or by decaying organic matter are common natural phenomena.

Scientific investigation of the luminescence phenomena began, when the Bolognian stone was discovered in 1603.

In 1603 - Vincenzo Casciarolo, a Bolognian shoemaker and an alchemist, prepared an artificial phosphor by accident. It was known as the Bolognian stone (or Bolognian phosphor) which glows after exposure to light.

In 1630 - Galileo Galilei (1564-1642), an Italian scientist, had the view on the Bolognian stone: "It must be explained how it happens that the light is conceived into the stone, and is given back after some time, as in childbirth."

In 1646- Athanasius Kircher, a German Jesuit priest recorded an interesting observation of the wood extract of Lignum nephriticum. An aqueous infusion of this wood exhibited blue color by reflected light and yellow color by transmitted light. The blue light is actually a type of light emission

(fluorescence) and therefore Kircher is often regarded as the "Discoverer of fluorescence".

In 1838- David Brewster, a Scottish preacher used the term "internal dispersion" to describe fluorescence phenomena.

In 1852- George Stokes, professor of mathematics and physics at Cambridge, interpreted the light-emitting phenomenon and formulated the law (the Stokes Law or the Stokes Shift) that the fluorescent light is of longer wavelength than the exciting light.

In 1853- Stokes coined the term "fluorescence" from the term "internal dispersion."

In 1856- William Perkin, an English chemist synthesized a coal-tar dye, aniline purple (the first synthetic dye). His breakthrough attracted the attention of numerous synthetic chemists and a variety of dyes were synthesized. Perkin was acknowledged as the Founder of the synthetic dye industry.

In 1864- Stokes lectured "On the application of the optical properties to detection and discrimination of organic substances" before the Chemical Society and the Royal Institution.

In 1871- Adolph Von Baeyer, a German chemist synthesized a fluorescent dye, fluoresceine.

In 1880- A German firm known as Dr. G. Greublers Chemisches Laboratorium started to test and package the most desirable dyes for biologists and medical researchers.

In 1882- Paul Erlich, a German bacteriologist employed the fluorescent dye uranin (sodium salt fluorescein) to track the pathway of secretion of

aqueous humor in the eye. This is the first case of the use of in vivo fluorochroming in animal physiology.

In 1884- The Gram stain, a method for staining bacteria, which contains gentian violet as an essential component was developed by Hans Gram, a Danish physician.

In 1887- Karl Noack, a professor in Geissen published a book listing about 660 fluorescent compounds, arranged according to the color of their fluorescent light.

In 1897- Richard Meyer, a German chemist, introduced the term "fluorophores" for chemical groups with which fluorescence was associated.

In 1908- In Heinrich Kayser´s "Handbuch der Spectroscopie, vol. 4" Heinrich Knoen, a German physicist, arranged 1700 fluorescent compounds alphabetically with references to literature.

In 1911, 1913 - The first fluorescence microscope was developed by O. Heimstaedt, a German physicist, (1911) and H. Lehmann, a German physicist, (1913) as an outgrowth of the UV microscope (1901-1904). The instrument was used to investigate the autofluorescence of bacteria, protozoa, plant and animal tissues, and bio-organic substances such as albumin, elastin, and keratin.

In 1914- S. Von Provazek, a German protozoologist employed the fluorescence microscope to study dye binding to living cells. He stated that fluorochromes introduced into the cell effectively illuminate the partial functions of the cell in the dark field of the fluorescence microscope. This was a giant step forward in experimental cytology.

In 1929- Philipp Ellinger, a German pharmacologist, and August Hirt, a German anatomist, modified the fluorescence microscope so that it could be used to examine opaque specimens from most living organs. The new instrument was called an "intravital microscope" and is considered as the first epi-fluoresence (or incident-light excitation) microscope.

Fluorescence: The Phenomenon:

Fluorescence is the phenomenon in which absorption of light of a given wavelength by a fluorescent molecule is followed by the emission of light at longer wavelengths.

The distribution of wavelength-dependent intensity that causes fluorescence is known as the fluorescence excitation spectrum, and the distribution of wavelength-dependent intensity of emitted energy is known as the fluorescence emission spectrum.

Fluorescence detection has three major advantages over other light-based investigation methods: high sensitivity, high speed, and safety. The point of safety refers to the fact that samples are not affected or destroyed in the process, and no hazardous byproducts are generated.

Sensitivity is an important issue because the fluorescence signal is proportional to the concentration of the substance being investigated. Relatively small changes in ion concentration in living cells can have significant physiological effects. Whereas absorbance measurements can reliably determine concentrations only as low as several tenths of a micromolar, fluorescence techniques can accurately measure one million times smaller -

pico- and even femto-molar concentrations. Quantities less than an attomole (<10-18 mole) may be detected.

Using fluorescence, one can monitor very rapid changes in concentration. Changes in fluorescence intensity on the order of picoseconds can be detected if necessary.

Because it is a non-invasive technique, fluorescence does not interfere with a sample. The excitation light levels required to generate a fluorescence signal are low, reducing the effects of photo-bleaching, and living tissue can be investigated with no adverse effects on its natural physiological behavior.

Fluorescence Lifetime Applications:

In the last twenty years, fluorescence spectroscopy has evolved into a powerful tool for the study of chemical, semiconductor, photochemical, and biochemical species. It can provide insight into such intimate processes as solvent-solute interactions, the structure and dynamics of nucleic acids, and the permeability of membranes.

Many of these measurements are made possible by the fluorescence lifetime, the average time that a molecule spends in the excited state before emitting a photon and returning to the ground state. It is an important and unique feature of an excited state.

The lifetime of fluorescence is very short. Most fluorescence lifetimes fall within the range of hundreds of picoseconds to hundreds of nanoseconds. The fluorescence lifetime can function as a molecular stopwatch, to observe a variety of interesting molecular events. An antibody may rotate slightly within its molecular environment. A protein can change orientation. A critical binding

reaction may occur. Because the time-scale of these events is similar to the fluorescence lifetime, the measurement of the fluorescence lifetime allows the researcher to peer into the molecule and observe these phenomena.

Uses of Autofluorescence in Biochemistry and Medicine:

- *Protein structure and folding*
- *Protein-antibody interactions*
- *Donor-acceptor distances*
- *Enzyme conformation in proteins and membranes*
- *Dynamics and structure of membranes*
- *Permeability and ion transport in membranes*
- *Lipid dynamics in membranes*
- *Dynamics and structure of nucleic acids*
- *Photochemistry of vision*
- *Mechanism of photosynthesis*
- *Photodynamic therapy*

Studies on autofluorescence techniques:

Liang et al (2000) [181] reported that most of the emissions are due to excitation of tryptophan residues, with a few emissions due to residues of tyrosine and phenyl alanine. Due to the higher fluorescence quantum yield of tryptophan, resonance energy transfer from proximal phenyl alanine to tyrosine and from tyrosine to tryptophan, the emission spectrum of tissues containing the three residues usually resembles that of tryptophan. Further, the photochemical characteristics of tryptophan are very much dependent on its

microenvironmental conditions. In particular, the emission of tryptophan depends upon its solvent polarity. The fluorescence spectrum shifts to shorter wavelength as the polarity of the solvents surrounding the tryptophan residues decreases.

Ueda Y and Kobayashi M. et al (2004) [182] observed that as the lactic-acid concentration becomes dense, the AF peak intensity from elastin and desmosine solutions become wholly weak. They found a similar reduction in the autofluorescence intensity for nicotinamide adenine dinucleotide (NADH) solutions. Their analysis indicated that the lactic acid causes the conformational change in elastin and the oxidation of NADH, which can be related to changes in the AF properties.

Autofluorescence in oral carcinoma:

Kolli VR et al (1995) [183], Chen CT et al (1996) [184], Dhingra JK et al (1996) [185], Ganesan S et al (1998)[186], Vengadasan et al (1998) [187], Chen CT et al (1998) [184], Gillenwater A et al (1998)[188], Lezlee Coghlan et al (2001)[189], Wei Zheng et al (2002) [190,] Madhuri S et al (2003) [191,] Diana C.G, de Veld et al (2003) [192] and Majumder. S. K et al (2003) [193] have reported various findings of autofluorescence in pre-cancer and oral cancer..

Autofluorescence of oral submucous fibrosis:

Hsin-ming chen et al (2003) [194] measured the in vivo autofluorescence spectra of 59 oral submucous fibrosis mucosal sites and compared the measured spectra with autofluorescence spectra obtained from 15 normal oral mucosal samples from 15 healthy volunteers, 5 samples of frictional keratosis on OSF (FHOSF) buccal mucosa and 29 samples of oral leukoplakia on OSF (OLOSF)

buccal mucosa. They found that the spectrum of the OSF mucosa had a significantly higher 380nm emission peak and a significantly lower 460nm emission peak than the spectra of NOM, FHOSF and OLOSF samples. They concluded that OSF has a very unique pattern of autofluorescence spectrum which can be used for real-time diagnosis of OSF.

Tsai T et al (2003) [195] assessed the utility of autofluorescence as a diagnostic tool for oral squamous cell carcinoma in a high risk population. They characterized the in vivo autofluorescence spectra from oral submucous fibrosis (OSF) lesions and oral premalignant and malignant lesions in both OSF and non-OSF patients. The mean ratio values increased gradually from OSF to NOM, to EH and ED, and to SCC. Their ANOVA test showed significant differences in the ratio value among all categories of samples ($P<0.01$). They found that EH, ED, and SCC lesions on OSF patients had distorted autofluorescence intensity because of collagen. While the mean ratio values of EH, ED, and SCC between non-OSF and OSF patients showed significant differences.

Wang CY et al (2003) [196] used a fiber optics-based fluorospectrometer to measure the autofluorescence spectra from healthy volunteers (NOM) and patients with oral lesions of submucous fibrosis (OSF), epithelial hyperkeratosis (EH), epithelial dysplasia (ED), and squamous cell carcinoma (SCC). They concluded that the PLS-ANN classification algorithm based on autofluorescence spectroscopy at 330-nm excitation is useful for in vivo diagnosis of OSF as well as oral premalignant and malignant lesions.

Electron microscopical findings:

Zorzetto (2002) [197, 198] studied the ultrastructural features of the cheek oral mucosa of rats (Rattus norvegicus) submitted to experimental chronic alcoholism by transmission electron microscopy. Samples of the oral mucosa from the cheek region were dissected and processed for ultrastructural analysis. The results showed cell alterations in the basal and intermediate layer of the mucosal epithelium. The most frequent alterations observed were an increased intercellular space, the presence of lipid droplets in the cytoplasm, and irregular nuclei with a pyknotic aspect.

Cheng LH and Hudson J (2002) [199] have used Transmission electron microscopy (TEM) to identify the ultrastructural details of normal and cancerous human oral mucosa and features of dysplasia for diagnostic purposes. Normal mucosa, severe dysplasia, oral SCC and normal margin adjacent to oral SCC were used to compare the ultrastructural features of normal and premalignant oral mucosa and oral SCC. Thin and discontinuous basal laminas were found in mucosa with severe dysplasia and normal margin adjacent to oral SCC. No basal lamina was identified in oral SCC. This study showed that there are some ultrastructural changes during malignant transformation of oral mucosa.

Kannan et al (1996) [200] assessed 15 biopsies sampled from the immediate adjacent epithelium of oral squamous cell carcinoma, under light and electron microscopy. Light microscopic examination of one micron thick sections revealed that the majority of lesions (67%) had hyperplastic or mildly dysplastic epithelium while the remaining (33%) had moderate to severe dysplasia. Ultrastructural observations showed that all these lesions had subcellular

alterations similar to those seen in frank malignant oral tissue, particularly in the lower half of the epithelium. Important ultrastructural changes observed included bizarre nuclei of basal and lower spinal cells, enlarged and multiple nucleoli, presence of inter-chromatin and perichromatin granules, loss of desmosomes and marked spongiosis as well as disturbed cellular maturation sequences in the keratinocytes evidenced by abnormal and irregular distribution of maturation markers such as keratohyalin granules and tonofilaments.

Kartha et al (1994) [201] studied the biopsies of twenty-five oral carcinomas and five normal oral epithelium, using light and electron microscopy. All histological types (well differentiated squamous cell carcinoma, moderately differentiated squamous cell carcinoma, poorly differentiated squamous cell carcinoma, verrucous carcinoma and spindle cell carcinoma) were seen in the study sample. In addition, 1 case of carcinoma in situ was also present. The normal oral epithelium consisted of three keratinizing types (gingiva) and two non-keratinizing types (buccal mucosa). The ultrastructural features of oral carcinomas showed good correlation with the features seen in light microscopy. The differentiation status of the lesions showed a relationship with cell and nuclear size, tonofilament and keratin content as well as few other cellular abnormalities. It was also observed that the fine details revealed by electron microscopy were often a means of explaining the characteristic histopathological features of oral carcinoma

Saito H and Itoh I (1993) [202] observed the ultrastructure on the upper and lower surfaces of buccal epithelial superficial layer cells and the arrangement of

intercellular junction apparatuses by scanning and transmission electron microscopy. In the superficial layer cells of the rabbit buccal epithelium, microvillus-like processes were observed on the lower surface and micro ridges on the upper surface. The distal portion of the microvillus-like process on the lower cell surface formed a wedge-like structure. At the inter-micro ridge areas on the upper cell surface, wedge-like depressions were observed. These depressions were interdigitated with the wedge-like structures of the microvillus-like processes. From the observation of the serial ultrathin sections, most interdigitation sites have two desmosomes per microvillus-like process (89.5%). The desmosomes were located in the distal portion of the microvillus-like processes (99.3%). The wedge-like structures of the microvillus-like processes and wedge-like depressions on the inter-micro ridge areas seem to increase the junctional surface and enhance cell cohesion against external forces from various directions.

Mascres C and Joly G (1981) [203] studied the action of alcohol on the oral mucosa of Sprague-Dawley female rats, histochemically and ultrastructurally. In the alcoholic animals, the epithelium was thickened from day 98 on and SS and SH groups were detected in the keratin; at this time interval, positive acid phosphatase areas were noted. Under the electron microscope, myelin figures were observed and phosphatase areas were noted. Under the electron microscope, myelin figures were observed between cells on days 98 and 200. Cells undergoing autolysis could explain the lysosomal enzyme activity; the DNA activity assessed by methyl-pyronine green stain was increased after 200 and 290 days. This activity was correlated at these time intervals with the

increase of the mitosis number; succino-dehydrogenase showed an increased activity on days 200 and 290. The mitochondria often of large size showed no ultrastructural changes even on day 290; the vessels, already on day 98, then on days 200 and 290, showed a decrease in alkaline phosphatase reactivity with occasional thinning of the wall. These modifications suggested that alcohol was able to modify the integrity of oral mucosa cells in the rat, perhaps by modifying the quality of the cell walls.

McKinney RV Jr, Singh BB (1977) [204] explored the morphologic nature of the basement membrane under neoplastic oral mucous membrane and the progression of changes associated with dysplasia, carcinoma in situ, and invasive carcinoma. They proposed that neoplastic epithelial cells may produce a collagenolytic enzyme which is released into the epithelial intercellular spaces. This enzyme permeates to the basement membrane, causes breaks in the basement membrane, and focal loss of stromal area contiguous with intercellular spaces. The neoplastic basal cells develop pseudopodia that eventually extend through the breaks in the basement membrane. This concept suggests that the basement membrane changes herald the progression of carcinoma in situ to invasive carcinoma.

Gao et al (1995) [205] performed a morphometric analysis of the spinous cell in 16 specimens of oral submucous fibrosis (OSF) by using interactive image analysis system (IBAS-II). 19 parameters of the size and shape were chosen, and compared with normal mucosa, leukoplakia, dysplasia and carcinoma. The results indicated that the cell dimensions (area, perimeter, all kinds of diameter) and nuclear cytoplasmic ratio in OSF were between normal mucosa and

dysplasia as well as carcinoma. The former showed a progressive decrease ($P < 0.01$), and the latter showed a progressive increase ($P < 0.01$). The dimensions of the nuclei did not show considerable differences among the groups ($P < 0.05$). The decrease of cell area and the increase of nuclear cytoplasmic ratio could reflect a malignant progress. The cell morphometric model could discriminate OSF well from other groups, suggesting that the change of the epithelium in OSF appearing in the spinous cell is specific itself.

MATERIALS & METHODS

MATERIALS AND METHODS

5o patients from the Department of Oral Medicine and Radiology of Saveetha Dental College and Hospitals, Chennai were taken for the present study.

The patients were categorized into the following groups,

- *GROUP I: 10 patients between the age group of 16 to 60 years, without the habits of smoking or alcoholism or pan chewing and without any oral lesions, as the controls.*
- *GROUP II: 10 patients between the age group of 16 to 60 years, with the habit of pan chewing (10-15 quid's per day) for about 5-10 years, without any oral lesions.*
- *GROUP III: 10 patients between the age group of 16 – 60 years, with the habits of smoking more than 10 cigarettes per day for about 5-10 years, without any oral lesions.*
- *GROUP IV: 5 patients with homogeneous leukoplakia involving the buccal mucosa.*
- *GROUP V: 5 patients with oral sub mucous fibrosis*
- *GROUP VI: 5 patients with well differentiated squamous cell carcinoma involving the buccal mucosa and alveolar ridge.*
- *GROUP VII: 5 patients with moderately differentiated squamous cell carcinoma involving the buccal mucosa and alveolar ridge.*
- *GROUP VIII: 5 patients with verrucous carcinoma.*

2. Autofluorescence technique:

Fluorescence spectroscopy was carried out using FLUOROMAX-2 (USA, 1996) in the Department of Medical physics at Anna University, Chennai.

In vivo fluorescence spectroscopy:

After clinical diagnosis, the patients of group I, II and III were taken to the Department of Medical Physics, Anna University, Chennai for in-vivo fluorescence spectroscopy.

The patient was seated comfortably in a chair. Fiber-optic probe attached to spectrofluorometer was placed in contact with the mucosa to record the data.

Readings in case of pan chewers were taken from the area where the quid was placed. Readings from the control group were taken from an area adjacent to the premolars at occlusal level.

Autofluorescence spectroscopy consists of two procedures:

1. *Excitation spectroscopy.*
2. *Emission spectroscopy.*

In excitation spectroscopy, the tissues are excited at a particular wavelength and the resulting emissions from the tissues are measured. In the present study, excitation spectroscopy was performed at 280 and 320nm respectively.

The second part consists of emission spectroscopy. Emission spectroscopy is a complementary technique, performed to confirm the findings of excitation spectroscopy and also to observe any conformational changes in the tissues. Emission spectroscopy was performed at 340 and 390nm excitation.

BIOPSY:

Subsequent to the autofluorescence spectroscopy, after taking informed consent, incisional biopsy was performed from test groups (n=5) and controls (n=5) under local anesthesia and the specimen was sent for histopathological evaluation and transmission electron microscopy.

Histopathological procedures:

The biopsy samples were rinsed in saline and then fixed with 10% buffered formalin solution. The tissue was processed routinely and embedded in paraffin. 4μ sections were prepared with a Leica semi-automated microtome (RM 2245).

Routine Hematoxylin and Eosin staining was done for microscopic examination of the sections. Sections were also prepared for special staining procedures to demonstrate collagen. These slides were stained with Van Gieson stain to demonstrate collagen in the tissue.

Staining protocol: Van Gieson Technique: (Van Gieson 1899)

Solution:

Saturated aqueous picric acid solution	*50 ml*
1% aqueous acid fuchsin solution	*9 ml*
Distilled water	*50 ml*

Method:

1. *Deparaffinize sections and bring to water.*
2. *Stain nuclei with Hematoxylin*
3. *Wash in tap water*
4. *Differentiate in acid alcohol*

5. *Wash well in tap water*
6. *Stain in van Gieson solution for 3 minutes*
7. *Blot and dehydrate through alcohols.*
8. *Clear in xylene and mount in DPX.*

Histological assessment was performed on the study samples (n= 15) and controls (n=25). The samples were grouped into,

- *C1 to C5 = positive controls – patients who are non-users of tobacco*
- *S1 to S5 = smokers*
- *P1 to P5 = pan chewers*
- *L1 to L5 = leukoplakia*
- *F1 to F5 = sub mucous fibrosis*
- *W1 to W5 = negative controls - well differentiated squamous cell carcinoma*
- *M1 to M5 = negative controls - moderately differentiated squamous cell carcinoma*
- *V1 to V5 = negative controls - verrucous carcinoma*

The parameters that were assessed,

1. *Keratinization – ortho(o) /para (p) /non-keratinized (n)*
2. *Thickness – atrophy (a) /hyperplastic (h) /normal(n)*
3. *Epithelium - connective tissue interface: - normal (n)/ thickened (t)/ discontinuous (d)*

<u>Connective tissue – sub-epithelial region:</u>

4. *Density – minimal (1)/ moderate (2)/ increased (3)*

5. *Pattern – wavy (w)/ bundles(b)/ haphazard (m)/ parallel (p)/ stream (s)*
6. *Fibroblasts - minimal (1)/ moderate (2)/ increased (3)*
7. *Inflammatory cells - absent (0)/minimal (1)/ moderate (2)/ intense (3)*
8. *Hyalinization – present (h)/ absent (n)*
9. *Vascularity - minimal (1)/ moderate (2)/ intense (3)*

Connective tissue – deeper region:

10. *Density – minimal (1)/ moderate (2)/ increased (3)*
11. *Pattern – wavy (w)/ bundles(b)/ haphazard (m)/ parallel (p)/ stream (s)*
12. *Fibroblasts - minimal (1)/ moderate (2)/ increased (3)*
13. *Inflammatory cells - absent (0)/minimal (1)/ moderate (2)/ intense (3)*
14. *Hyalinization – present (h)/ absent (n)*
15. *Vascularity - minimal (1)/ moderate (2)/ intense (3)*
16. *Dysplasia – absent (o)/ present (d)*
17. *Malignant islands – absent (0)/ present (1)*
18. *Connective tissue status - homogenous (h)/ lysis (l)*
19. *Van Gieson stain intensity - minimal (1)/ moderate (2)/ intense (3)*

Electron microscopy:

Five representative samples were fixed in glutaraldehyde, processed and subjected to transmission electron microscopy. The protocol is as follows,

1. *The tissue samples were fixed with 2.5% buffered glutaraldehyde at 4^{o}C overnight.*
2. *It was then washed with cacodylate buffer (pH-7.2) for 15 minutes.*
3. *Post fixation was done with 1% buffered osmium tetroxide for 2 hours at 4^{o}C*

4. *Washed in buffer for 10 minutes.*
5. *Dehydrated in ascending grades of alcohol (30%, 50%, 70%, 80%, 90% and 100%) for 10 minutes in each concentration.*
6. *Treated with propylene oxide for 10 minutes.*
7. *Infiltrated with resin-propylene oxide mixture in the ratio – 1:3 initially for two hours, later reverted to 3:1 ratio for two hours and finally in 100% resin for two hours.*
8. *Embedded with epoxy resin (EPON 812 polar bed resin) with catalyst and incubated at 60^{o}C for 48 hours.*
9. *Blocks were made, serial sections done and stained with toluidine blue for light microscopy.*
10. *The site was located on light microscopy and sections of 1 micron thickness was prepared with Leica ultracut microtome.*
11. *Silver grey sections were collected on copper grids and stained with saturated solution of uranyl acetate and lead citrate.*
12. *Sections were scanned with JEOL 100 SX electron microscope with an acceleration voltage of 60 to 80 KV.*
13. *Selected areas were then photographed.*

All the parameters were tabulated as shown in the master chart (fig.1) and the data were fed into a computer and the statistical significance was assessed with SPSS Version 10.

STATISTICS

Histological assessment of the samples:

Comparison of Controls, Smokers and Pan Chewers:

Group * Keratinization

Crosstab

			Keratinisation			Total
			O	P	N	
Group	C	Count	2	3		5
		% within Gro	40.0%	60.0%		100.0%
	S	Count		4	1	5
		% within Gro		80.0%	20.0%	100.0%
	P	Count		5		5
		% within Gro		100.0%		100.0%
Total		Count	2	12	1	15
		% within Gro	13.3%	80.0%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig (2-sided)
Pearson Chi-Sq	6.500[a]	4	.165
Likelihood Ratio	7.097	4	.131
Linear-by-Linear Association	1.909	1	.167
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count le minimum expected count is .33.

Group * Thickness

Crosstab

			Thickness	Total
			H	
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	. [a]
N of Valid Cases	15

a. No statistics are computed because Thickness is a constant.

Group * Interface

Crosstab

			Interface		Total
			N	T	
Group	C	Count	3	2	5
		% within Gro	60.0%	40.0%	100.0%
	S	Count	3	2	5
		% within Gro	60.0%	40.0%	100.0%
	P	Count	4	1	5
		% within Gro	80.0%	20.0%	100.0%
Total		Count	10	5	15
		% within Gro	66.7%	33.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig (2-sided)
Pearson Chi-Sq	.600[a]	2	.741
Likelihood Ratio	.631	2	.729
Linear-by-Linear Association	.420	1	.517
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less minimum expected count is 1.67.

Group * Connective tissue - Subepithelial - Density

Crosstab

			CT - Subep - Density			
			+	++	+++	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
Total		Count	5	9	1	15
		% within Group	33.3%	60.0%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.400[a]	4	.663
Likelihood Ratio	2.634	4	.621
Linear-by-Linear Association	1.135	1	.287
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Connective tissue - Subepithelial - Pattern

Crosstab

			CT - Subep - Pattern			
			Wavy	Bundles	Haphazard	Total
Group	C	Count	3	1	1	5
		% within Group	60.0%	20.0%	20.0%	100.0%
	S	Count		1	4	5
		% within Group		20.0%	80.0%	100.0%
	P	Count	3		2	5
		% within Group	60.0%		40.0%	100.0%
Total		Count	6	2	7	15
		% within Group	40.0%	13.3%	46.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig (2-sided)
Pearson Chi-Sq	6.000[a]	4	.199
Likelihood Ratio	8.488	4	.075
Linear-by-Linear Association	.108	1	.742
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less minimum expected count is .67.

Group * Connective tissue- Subepithelial - Fibroblasts

Crosstab

			CT - Subep - Fibroblasts		
			+	++	Total
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	12	3	15
		% within Group	80.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squar	2.500[a]	2	.287
Likelihood Ratio	3.278	2	.194
Linear-by-Linear Association	.583	1	.445
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. T minimum expected count is 1.00.

Group * Connective tissue - Subepithelial – Inflammatory cells

Crosstab

			CT - Subep - Inflammatory			
			-	+	++	Total
Group	C	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	S	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	P	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
Total		Count	3	10	2	15
		% within Group	20.0%	66.7%	13.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squa	7.800[a]	4	.099
Likelihood Ratio	9.087	4	.059
Linear-by-Linear Association	4.541	1	.033
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less thar minimum expected count is .67.

Group * Connective tissue - Subepithelial - Hyalinization

Crosstab

			CT - Subep - Hyalinization	
			Negative	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Squa	.[a]
N of Valid Cases	15

a. No statistics are computed because - Subep - Hyalinisation is a constan

Group * Connective tissue - Subepithelial - Vascularity

Crosstab

			CT - Subep - Vascularity		
			+	++	Total
Group	C	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	S	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	P	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	9	6	15
		% within Group	60.0%	40.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squ	1.667[a]	2	.435
Likelihood Ratio	1.726	2	.422
Linear-by-Linear Association	.389	1	.533
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less tl minimum expected count is 2.00.

Group * Connective tissue – Deeper region - Density

Crosstab

			CT - Deep - Density			
			+	++	+++	Total
Group	C	Count	1	2	2	5
		% within Group	20.0%	40.0%	40.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
Total		Count	7	6	2	15
		% within Group	46.7%	40.0%	13.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squar	7.000[a]	4	.136
Likelihood Ratio	7.442	4	.114
Linear-by-Linear Association	4.773	1	.029
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. minimum expected count is .67.

Group * Connective tissue - Deep - Pattern

Crosstab

			CT - Deep - Pattern			
			Wavy	Bundles	Haphazard	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	S	Count	1	1	3	5
		% within Group	20.0%	20.0%	60.0%	100.0%
	P	Count	3		2	5
		% within Group	60.0%		40.0%	100.0%
Total		Count	5	4	6	15
		% within Group	33.3%	26.7%	40.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squ	6.100[a]	4	.192
Likelihood Ratio	6.820	4	.146
Linear-by-Linear Association	.128	1	.720
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less t minimum expected count is 1.33.

Group * Connective tissue - Deep - Fibroblasts

Crosstab

			CT - Deep - Fibroblasts		
			+	++	Total
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	5		5
		% within Group	100.0%		100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	14	1	15
		% within Group	93.3%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squa	2.143[a]	2	.343
Likelihood Ratio	2.344	2	.310
Linear-by-Linear Association	1.500	1	.221
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less tha minimum expected count is .33.

Group * Connective tissue - Deep - Inflammatory

Crosstab

			CT - Deep - Inflammatory				
			-	+	++	+++	Total
Group	C	Count	3	2			5
		% within Grou	60.0%	40.0%			100.0%
	S	Count	1	4			5
		% within Grou	20.0%	80.0%			100.0%
	P	Count		1	3	1	5
		% within Grou		20.0%	60.0%	20.0%	100.0%
Total		Count	4	7	3	1	15
		% within Grou	26.7%	46.7%	20.0%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squ	13.500[a]	6	.036
Likelihood Ratio	15.080	6	.020
Linear-by-Linear Association	8.195	1	.004
N of Valid Cases	15		

a. 12 cells (100.0%) have expected count less minimum expected count is .33.

Group * Connective tissue - Deep - Hyalinization

Crosstab

			CT - Deep - Hyalinisation	
			Negative	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Squa	.[a]
N of Valid Cases	15

a. No statistics are computed becaus - Deep - Hyalinisation is a constan

Group * Connective tissue - Deep - Vascularity

Crosstab

			CT - Deep - Vascularity		
			+	++	Total
Group	C	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
Total		Count	8	7	15
		% within Group	53.3%	46.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squa	.536[a]	2	.765
Likelihood Ratio	.537	2	.764
Linear-by-Linear Association	.375	1	.540
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less tha minimum expected count is 2.33.

Group * Dysplasia

Crosstab

			Dysplasia	
			-	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	15

a. No statistics are computed because Dysplatia is a constant

Group * Malignant Islands

Crosstab

			Malignant Islands	
			-	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	15

a. No statistics are computed because Malignant Islands is a constant.

Group * Status of connective tissue - Homogenous / Lysis

Crosstab

			Homogenous / Lysis		
			H	L	Total
Group	C	Count	5		5
		% within Group	100.0%		100.0%
	S	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	12	3	15
		% within Group	80.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.500[a]	2	.024
Likelihood Ratio	8.282	2	.016
Linear-by-Linear Association	.000	1	1.000
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Vangieson

Crosstab

			Vangieson			
			+	++	+++	Total
Group	C	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
	S	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	P	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
Total		Count	2	12	1	15
		% within Group	13.3%	80.0%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.000[a]	4	.558
Likelihood Ratio	3.819	4	.431
Linear-by-Linear Association	.000	1	1.000
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Comparison of Controls, Smokers, Pan chewers, Leukoplakia, Sub mucous fibrosis, Well differentiated squamous cell carcinoma, Moderately differentiated squamous cell carcinoma and Verrucous carcinoma:

Group * Keratinization

Crosstab

			Keratinisation			
			O	P	N	Total
Group	C	Count	2	3		5
		% within Gro	40.0%	60.0%		100.0%
	S	Count		4	1	5
		% within Gro		80.0%	20.0%	100.0%
	P	Count		5		5
		% within Gro		100.0%		100.0%
	L	Count		5		5
		% within Gro		100.0%		100.0%
	F	Count	3	2		5
		% within Gro	60.0%	40.0%		100.0%
	W	Count		5		5
		% within Gro		100.0%		100.0%
	M	Count		5		5
		% within Gro		100.0%		100.0%
	V	Count		5		5
		% within Gro		100.0%		100.0%
Total		Count	5	34	1	40
		% within Gro	12.5%	85.0%	2.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squ	25.035[a]	14	.034
Likelihood Ratio	20.759	14	.108
Linear-by-Linear Association	.298	1	.585
N of Valid Cases	40		

a. 24 cells (100.0%) have expected count less minimum expected count is .13.

Group * Thickness

Crosstab

			Thickness		
			A	H	Total
Group	C	Count		5	5
		% within Group		100.0%	100.0%
	S	Count		5	5
		% within Group		100.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	L	Count		5	5
		% within Group		100.0%	100.0%
	F	Count	5		5
		% within Group	100.0%		100.0%
	W	Count		5	5
		% within Group		100.0%	100.0%
	M	Count		5	5
		% within Group		100.0%	100.0%
	V	Count		5	5
		% within Group		100.0%	100.0%
Total		Count	5	35	40
		% within Group	12.5%	87.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-S	40.000[a]	7	.000
Likelihood Rati	30.142	7	.000
Linear-by-Linear Association	.265	1	.606
N of Valid Cas	40		

a. 16 cells (100.0%) have expected coun minimum expected count is .63.

Group * Interface

Crosstab

			Interface			
			N	T	D	Total
Group	C	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	S	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	P	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	L	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	F	Count	5			5
		% within Group	100.0%			100.0%
	W	Count			5	5
		% within Group			100.0%	100.0%
	M	Count			5	5
		% within Group			100.0%	100.0%
	V	Count	5			5
		% within Group	100.0%			100.0%
Total		Count	24	6	10	40
		% within Group	60.0%	15.0%	25.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	46.667[a]	14	.000
Likelihood Ratio	51.543	14	.000
Linear-by-Linear Association	4.314	1	.038
N of Valid Cases	40		

a. 24 cells (100.0%) have expected count less than 5. The minimum expected count is .75.

Group * Connective tissue - Subepithelial - Density

Crosstab

			CT - Subep - Dentity			
			+	++	+++	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	L	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	F	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	W	Count	5			5
		% within Group	100.0%			100.0%
	M	Count	5			5
		% within Group	100.0%			100.0%
	V	Count	5			5
		% within Group	100.0%			100.0%
Total		Count	26	13	1	40
		% within Group	65.0%	32.5%	2.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squ	22.769[a]	14	.064
Likelihood Ratio	24.304	14	.042
Linear-by-Linear Association	13.261	1	.000
N of Valid Cases	40		

a. 24 cells (100.0%) have expected count less th
minimum expected count is .13.

Group * Connective tissue - Subepithelial - Pattern

Crosstab

			CT - Subep - Pattern					Total
			Wavy	Bundles	Haphazard	Parallel	Stream	
Group	C	Count	3	1	1			5
		% within Group	60.0%	20.0%	20.0%			100.0%
	S	Count		1	4			5
		% within Group		20.0%	80.0%			100.0%
	P	Count	3		2			5
		% within Group	60.0%		40.0%			100.0%
	L	Count	1		4			5
		% within Group	20.0%		80.0%			100.0%
	F	Count			1	4		5
		% within Group			20.0%	80.0%		100.0%
	W	Count	1		4			5
		% within Group	20.0%		80.0%			100.0%
	M	Count			3		2	5
		% within Group			60.0%		40.0%	100.0%
	V	Count			5			5
		% within Group			100.0%			100.0%
Total		Count	8	2	24	4	2	40
		% within Group	20.0%	5.0%	60.0%	10.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	65.333[a]	28	.000
Likelihood Ratio	49.678	28	.007
Linear-by-Linear Association	9.034	1	.003
N of Valid Cases	40		

a. 40 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Connective tissue - Subepithelial – Fibroblasts:

Crosstab

			CT - Subep - Fibroblasts		Total
			+	++	
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	L	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	W	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	M	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	V	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
Total		Count	27	13	40
		% within Group	67.5%	32.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squ	7.179[a]	7	.410
Likelihood Ratio	8.514	7	.289
Linear-by-Linear Association	2.799	1	.094
N of Valid Cases	40		

a. 16 cells (100.0%) have expected count less minimum expected count is 1.63.

Group * Connective tissue - Subepithelial – Inflammatory cells

Crosstab

			CT - Subep - Inflammatory				
			-	+	++	+++	Total
Group	C	Count	3	2			5
		% within Group	60.0%	40.0%			100.0%
	S	Count		4	1		5
		% within Group		80.0%	20.0%		100.0%
	P	Count		4	1		5
		% within Group		80.0%	20.0%		100.0%
	L	Count		1		4	5
		% within Group		20.0%		80.0%	100.0%
	F	Count		3	2		5
		% within Group		60.0%	40.0%		100.0%
	W	Count		2		3	5
		% within Group		40.0%		60.0%	100.0%
	M	Count		1	3	1	5
		% within Group		20.0%	60.0%	20.0%	100.0%
	V	Count			1	4	5
		% within Group			20.0%	80.0%	100.0%
Total		Count	3	17	8	12	40
		% within Group	7.5%	42.5%	20.0%	30.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	52.000[a]	21	.000
Likelihood Ratio	49.571	21	.000
Linear-by-Linear Association	15.611	1	.000
N of Valid Cases	40		

a. 32 cells (100.0%) have expected count less than 5. The minimum expected count is .38.

Group * Connective tissue - Subepithelial - Hyalinization

Crosstab

			CT - Subep - Hyalinisation		
			Present	Negative	Total
Group	C	Count		5	5
		% within Group		100.0%	100.0%
	S	Count		5	5
		% within Group		100.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	L	Count		5	5
		% within Group		100.0%	100.0%
	F	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	W	Count		5	5
		% within Group		100.0%	100.0%
	M	Count		5	5
		% within Group		100.0%	100.0%
	V	Count		5	5
		% within Group		100.0%	100.0%
Total		Count	3	37	40
		% within Group	7.5%	92.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig (2-sided)
Pearson Chi-Sq	22.703[a]	7	.002
Likelihood Ratio	14.581	7	.042
Linear-by-Linear Association	.151	1	.698
N of Valid Case	40		

a. 16 cells (100.0%) have expected count le minimum expected count is .38.

Group * Connective tissue - Subepithelial - Vascularity

Crosstab

			CT - Subep - Vascularity			
			+	++	+++	Total
Group	C	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	L	Count	2	2	1	5
		% within Group	40.0%	40.0%	20.0%	100.0%
	F	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	W	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	M	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	V	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
Total		Count	24	14	2	40
		% within Group	60.0%	35.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	12.476[a]	14	.568
Likelihood Ratio	12.369	14	.577
Linear-by-Linear Association	.214	1	.644
N of Valid Cases	40		

a. 24 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Connective tissue - Deep - Density

Crosstab

			CT - Deep - Dentity			
			+	++	+++	Total
Group	C	Count	1	2	2	5
		% within Group	20.0%	40.0%	40.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	L	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	F	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	W	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	M	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	V	Count	5			5
		% within Group	100.0%			100.0%
Total		Count	25	13	2	40
		% within Group	62.5%	32.5%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig (2-sided)
Pearson Chi-S	22.966[a]	14	.061
Likelihood Rati	18.954	14	.167
Linear-by-Linea Association	7.445	1	.006
N of Valid Case	40		

a. 24 cells (100.0%) have expected count minimum expected count is .25.

Group * Connective tissue - Deep - Pattern

Crosstab

			CT - Deep - Pattern				Total
			Wavy	Bundles	Haphazard	Stream	
Group	C	Count	1	3	1		5
		% within Group	20.0%	60.0%	20.0%		100.0%
	S	Count	1	1	3		5
		% within Group	20.0%	20.0%	60.0%		100.0%
	P	Count	3		2		5
		% within Group	60.0%		40.0%		100.0%
	L	Count	1		4		5
		% within Group	20.0%		80.0%		100.0%
	F	Count			5		5
		% within Group			100.0%		100.0%
	W	Count	1		4		5
		% within Group	20.0%		80.0%		100.0%
	M	Count			3	2	5
		% within Group			60.0%	40.0%	100.0%
	V	Count			5		5
		% within Group			100.0%		100.0%
Total		Count	7	4	27	2	40
		% within Group	17.5%	10.0%	67.5%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	41.968[a]	21	.004
Likelihood Ratio	33.556	21	.040
Linear-by-Linear Association	9.333	1	.002
N of Valid Cases	40		

a. 32 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Connective tissue - Deep - Fibroblasts

Crosstab

			CT - Deep - Fibroblasts		Total
			+	++	
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	5		5
		% within Group	100.0%		100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	L	Count	5		5
		% within Group	100.0%		100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	W	Count	1	4	5
		% within Group	20.0%	80.0%	100.0%
	M	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	V	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
Total		Count	28	12	40
		% within Group	70.0%	30.0%	100.0%

Chi-Square Tests

	Value	df	symp. Sig (2-sided)
Pearson Chi-S	17.143[a]	7	.016
Likelihood Ra	20.397	7	.005
Linear-by-Line Association	9.750	1	.002
N of Valid Cas	40		

a. 16 cells (100.0%) have expected co minimum expected count is 1.50.

Group * Connective tissue - Deep - Inflammatory

Crosstab

			CT - Deep - Inflammatory				Total
			-	+	++	+++	
Group	C	Count	3	2			5
		% within Group	60.0%	40.0%			100.0%
	S	Count	1	4			5
		% within Group	20.0%	80.0%			100.0%
	P	Count		1	3	1	5
		% within Group		20.0%	60.0%	20.0%	100.0%
	L	Count		3	1	1	5
		% within Group		60.0%	20.0%	20.0%	100.0%
	F	Count		3	2		5
		% within Group		60.0%	40.0%		100.0%
	W	Count		1		4	5
		% within Group		20.0%		80.0%	100.0%
	M	Count			4	1	5
		% within Group			80.0%	20.0%	100.0%
	V	Count		1	2	2	5
		% within Group		20.0%	40.0%	40.0%	100.0%
Total		Count	4	15	12	9	40
		% within Group	10.0%	37.5%	30.0%	22.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	44.978[a]	21	.002
Likelihood Ratio	45.564	21	.001
Linear-by-Linear Association	14.862	1	.000
N of Valid Cases	40		

a. 32 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

Group * Connective tissue - Deep – Hyalinization

Crosstab

			CT - Deep - Hyalinisation	Total
			Negative	
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	L	Count	5	5
		% within Group	100.0%	100.0%
	F	Count	5	5
		% within Group	100.0%	100.0%
	W	Count	5	5
		% within Group	100.0%	100.0%
	M	Count	5	5
		% within Group	100.0%	100.0%
	V	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	40	40
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Squa	.[a]
N of Valid Cases	40

a. No statistics are computed becaus
- Deep - Hyalinisation is a constan

Group * Connective tissue - Deep - Vascularity

Crosstab

			CT - Deep - Vascularity			
			+	++	+++	Total
Group	C	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	S	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	P	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	L	Count	3	1	1	5
		% within Group	60.0%	20.0%	20.0%	100.0%
	F	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	W	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	M	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	V	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
Total		Count	23	16	1	40
		% within Group	57.5%	40.0%	2.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	11.696[a]	14	.631
Likelihood Ratio	8.993	14	.831
Linear-by-Linear Association	.000	1	1.000
N of Valid Cases	40		

a. 24 cells (100.0%) have expected count less than 5. The minimum expected count is .13.

Group * Dysplasia

Crosstab

			Dysplatia		
			+	-	Total
Group	C	Count		5	5
		% within Group		100.0%	100.0%
	S	Count		5	5
		% within Group		100.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	L	Count	5		5
		% within Group	100.0%		100.0%
	F	Count		5	5
		% within Group		100.0%	100.0%
	W	Count	5		5
		% within Group	100.0%		100.0%
	M	Count	5		5
		% within Group	100.0%		100.0%
	V	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	20	20	40
		% within Group	50.0%	50.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squa	40.000[a]	7	.000
Likelihood Ratio	55.452	7	.000
Linear-by-Linear Association	22.750	1	.000
N of Valid Cases	40		

a. 16 cells (100.0%) have expected count less thar minimum expected count is 2.50.

Group * Malignant Islands

Crosstab

			Malignant Islands		
			+	-	Total
Group	C	Count		5	5
		% within Group		100.0%	100.0%
	S	Count		5	5
		% within Group		100.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	L	Count		5	5
		% within Group		100.0%	100.0%
	F	Count		5	5
		% within Group		100.0%	100.0%
	W	Count	5		5
		% within Group	100.0%		100.0%
	M	Count	5		5
		% within Group	100.0%		100.0%
	V	Count		5	5
		% within Group		100.0%	100.0%
Total		Count	10	30	40
		% within Group	25.0%	75.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	40.000[a]	7	.000
Likelihood Ratio	44.987	7	.000
Linear-by-Linear Association	9.905	1	.002
N of Valid Cases	40		

a. 16 cells (100.0%) have expected count less than 5. The minimum expected count is 1.25.

Group * Homogenous / Lysis

Crosstab

			Homogenous / Lysis		
			H	L	Total
Group	C	Count	5		5
		% within Group	100.0%		100.0%
	S	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	L	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	W	Count	1	4	5
		% within Group	20.0%	80.0%	100.0%
	M	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	V	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	26	14	40
		% within Group	65.0%	35.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squ	13.626[a]	7	.058
Likelihood Ratio	16.593	7	.020
Linear-by-Linear Association	1.653	1	.199
N of Valid Cases	40		

a. 16 cells (100.0%) have expected count less minimum expected count is 1.75.

Group * Vangieson

Crosstab

			Vangieson			
			+	++	+++	Total
Group	C	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
	S	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	P	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
	L	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
	F	Count		1	4	5
		% within Group		20.0%	80.0%	100.0%
	W	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	M	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	V	Count		5		5
		% within Group		100.0%		100.0%
Total		Count	9	26	5	40
		% within Group	22.5%	65.0%	12.5%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	38.414[a]	14	.000
Likelihood Ratio	33.291	14	.003
Linear-by-Linear Association	.874	1	.350
N of Valid Cases	40		

a. 24 cells (100.0%) have expected count less than 5. The minimum expected count is .63.

Comparison of Controls, Smokers, Pan chewers and Leukoplakia:

Group * Keratinization

Crosstab

			Keratinisation			Total
			O	P	N	
Group	C	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	S	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	P	Count		5		5
		% within Group		100.0%		100.0%
	L	Count		5		5
		% within Group		100.0%		100.0%
Total		Count	2	17	1	20
		% within Group	10.0%	85.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	9.647[a]	6	.140
Likelihood Ratio	8.993	6	.174
Linear-by-Linear Association	1.610	1	.204
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Thickness

Crosstab

			Thickness	
			H	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	L	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	20	20
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Squ	.[a]
N of Valid Cases	20

a. No statistics are computed because Thickness is a const

Group * Interface

Crosstab

			Interface		
			N	T	Total
Group	C	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	L	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	14	6	20
		% within Group	70.0%	30.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.952[a]	3	.813
Likelihood Ratio	.966	3	.809
Linear-by-Linear Association	.724	1	.395
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 1.50.

Group * Connective tissue - Subepithelial - Density

Crosstab

			CT - Subep - Density			
			+	++	+++	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	L	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
Total		Count	9	10	1	20
		% within Group	45.0%	50.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.311[a]	6	.389
Likelihood Ratio	6.261	6	.395
Linear-by-Linear Association	4.024	1	.045
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Connective tissue - Subepithelial - Pattern

Crosstab

			CT - Subep - Pattern			Total
			Wavy	Bundles	Haphazard	
Group	C	Count	3	1	1	5
		% within Group	60.0%	20.0%	20.0%	100.0%
	S	Count		1	4	5
		% within Group		20.0%	80.0%	100.0%
	P	Count	3		2	5
		% within Group	60.0%		40.0%	100.0%
	L	Count	1		4	5
		% within Group	20.0%		80.0%	100.0%
Total		Count	7	2	11	20
		% within Group	35.0%	10.0%	55.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	8.312[a]	6	.216
Likelihood Ratio	10.819	6	.094
Linear-by-Linear Association	1.105	1	.293
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

Group * Connective tissue - Subepithelial - Fibroblasts

Crosstab

			CT - Subep - Fibroblasts		Total
			+	++	
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	L	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	16	4	20
		% within Group	80.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.500[a]	3	.475
Likelihood Ratio	3.278	3	.351
Linear-by-Linear Association	.238	1	.626
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Connective tissue - Subepithelial - Inflammatory

Crosstab

			CT - Subep - Inflammatory				
			-	+	++	+++	Total
Group	C	Count	3	2			5
		% within Group	60.0%	40.0%			100.0%
	S	Count		4	1		5
		% within Group		80.0%	20.0%		100.0%
	P	Count		4	1		5
		% within Group		80.0%	20.0%		100.0%
	L	Count		1		4	5
		% within Group		20.0%		80.0%	100.0%
Total		Count	3	11	2	4	20
		% within Group	15.0%	55.0%	10.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	25.455[a]	9	.003
Likelihood Ratio	24.879	9	.003
Linear-by-Linear Association	11.154	1	.001
N of Valid Cases	20		

a. 16 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

Group * Connective tissue - Subepithelial - Hyalinization

Crosstab

			CT - Subep - Hyalinisation	
			Negative	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	L	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	20	20
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	20

a. No statistics are computed because CT - Subep - Hyalinisation is a constant.

Group * Connective tissue - Subepithelial - Vascularity

Crosstab

			CT - Subep - Vascularity			
			+	++	+++	Total
Group	C	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	L	Count	2	2	1	5
		% within Group	40.0%	40.0%	20.0%	100.0%
Total		Count	11	8	1	20
		% within Group	55.0%	40.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.000[a]	6	.544
Likelihood Ratio	4.791	6	.571
Linear-by-Linear Association	.434	1	.510
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Connective tissue - Deep - Density

Crosstab

			CT - Deep - Dentity			
			+	++	+++	Total
Group	C	Count	1	2	2	5
		% within Group	20.0%	40.0%	40.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	L	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
Total		Count	11	7	2	20
		% within Group	55.0%	35.0%	10.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	10.026[a]	6	.124
Likelihood Ratio	9.773	6	.135
Linear-by-Linear Association	6.135	1	.013
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

Group * Connective tissue - Deep - Pattern

Crosstab

			CT - Deep - Pattern			
			Wavy	Bundles	Haphazard	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	S	Count	1	1	3	5
		% within Group	20.0%	20.0%	60.0%	100.0%
	P	Count	3		2	5
		% within Group	60.0%		40.0%	100.0%
	L	Count	1		4	5
		% within Group	20.0%		80.0%	100.0%
Total		Count	6	4	10	20
		% within Group	30.0%	20.0%	50.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	10.000[a]	6	.125
Likelihood Ratio	10.447	6	.107
Linear-by-Linear Association	.450	1	.502
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Connective tissue - Deep - Fibroblasts

Crosstab

			CT - Deep - Fibroblasts		
			+	++	Total
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	5		5
		% within Group	100.0%		100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	L	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	19	1	20
		% within Group	95.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.158[a]	3	.368
Likelihood Ratio	2.937	3	.402
Linear-by-Linear Association	1.800	1	.180
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Connective tissue - Deep - Inflammatory

Crosstab

			CT - Deep - Inflammatory				
			-	+	++	+++	Total
Group	C	Count	3	2			5
		% within Group	60.0%	40.0%			100.0%
	S	Count	1	4			5
		% within Group	20.0%	80.0%			100.0%
	P	Count		1	3	1	5
		% within Group		20.0%	60.0%	20.0%	100.0%
	L	Count		3	1	1	5
		% within Group		60.0%	20.0%	20.0%	100.0%
Total		Count	4	10	4	2	20
		% within Group	20.0%	50.0%	20.0%	10.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	16.000[a]	9	.067
Likelihood Ratio	18.085	9	.034
Linear-by-Linear Association	7.200	1	.007
N of Valid Cases	20		

a. 16 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

Group * CT - Deep - Hyalinization

Crosstab

			CT - Deep - Hyalinisation	Total
			Negative	
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	L	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	20	20
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	20

a. No statistics are computed because CT - Deep - Hyalinisation is a constant.

Group * Connective tissue - Deep - Vascularity

Crosstab

			CT - Deep - Vascularity			Total
			+	++	+++	
Group	C	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	S	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	P	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	L	Count	3	1	1	5
		% within Group	60.0%	20.0%	20.0%	100.0%
Total		Count	11	8	1	20
		% within Group	55.0%	40.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	4.273[a]	6	.640
Likelihood Ratio	4.111	6	.662
Linear-by-Linear Association	.000	1	1.000
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Dysplasia

Crosstab

			Dysplatia		
			+	-	Total
Group	C	Count		5	5
		% within Group		100.0%	100.0%
	S	Count		5	5
		% within Group		100.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	L	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	5	15	20
		% within Group	25.0%	75.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	20.000[a]	3	.000
Likelihood Ratio	22.493	3	.000
Linear-by-Linear Association	11.400	1	.001
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 1.25.

Group * Malignant Islands

Crosstab

			Malignant Islands	
			-	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	L	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	20	20
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	20

a. No statistics are computed because Malignant Islands is a constant.

Group * Homogenous / Lysis

Crosstab

			Homogenous / Lysis		
			H	L	Total
Group	C	Count	5		5
		% within Group	100.0%		100.0%
	S	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	L	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
Total		Count	15	5	20
		% within Group	75.0%	25.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.200[a]	3	.066
Likelihood Ratio	9.033	3	.029
Linear-by-Linear Association	.456	1	.499
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 1.25.

Group * Vangieson

Crosstab

			Vangieson			
			+	++	+++	Total
Group	C	Count	1	4		5
		% within Grou	20.0%	80.0%		100.0%
	S	Count		4	1	5
		% within Grou		80.0%	20.0%	100.0%
	P	Count	1	4		5
		% within Grou	20.0%	80.0%		100.0%
	L	Count	1	4		5
		% within Grou	20.0%	80.0%		100.0%
Total		Count	3	16	1	20
		% within Grou	15.0%	80.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig (2-sided)
Pearson Chi-S	4.000[a]	6	.677
Likelihood Rat	4.499	6	.610
Linear-by-Line Association	.200	1	.655
N of Valid Cas	20		

a. 12 cells (100.0%) have expected cour minimum expected count is .25.

Comparison between Controls, Smokers, Pan chewers and Sub mucous fibrosis:

Group * Keratinization

Crosstab

			Keratinisation			
			O	P	N	Total
Group	C	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	S	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	P	Count		5		5
		% within Group		100.0%		100.0%
	F	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
Total		Count	5	14	1	20
		% within Group	25.0%	70.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	9.829[a]	6	.132
Likelihood Ratio	11.377	6	.077
Linear-by-Linear Association	1.336	1	.248
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Thickness

Crosstab

			Thickness		
			A	H	Total
Group	C	Count		5	5
		% within Group		100.0%	100.0%
	S	Count		5	5
		% within Group		100.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	F	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	5	15	20
		% within Group	25.0%	75.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	20.000[a]	3	.000
Likelihood Ratio	22.493	3	.000
Linear-by-Linear Association	14.657	1	.000
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 1.25.

Group * Interface

Crosstab

			Interface		
			N	T	Total
Group	C	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	F	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	15	5	20
		% within Group	75.0%	25.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.933[a]	3	.402
Likelihood Ratio	4.029	3	.258
Linear-by-Linear Association	2.613	1	.106
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 1.25.

Group * Connective tissue - Subepithelial - Density

Crosstab

			CT - Subep - Dentity			
			+	++	+++	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	F	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
Total		Count	7	12	1	20
		% within Group	35.0%	60.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.429[a]	6	.753
Likelihood Ratio	3.256	6	.776
Linear-by-Linear Association	.858	1	.354
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Connective tissue - Subepithelial - Pattern

Crosstab

			CT - Subep - Pattern				
			Wavy	Bundles	Haphazard	Parallel	Total
Group	C	Count	3	1	1		5
		% within Group	60.0%	20.0%	20.0%		100.0%
	S	Count		1	4		5
		% within Group		20.0%	80.0%		100.0%
	P	Count	3		2		5
		% within Group	60.0%		40.0%		100.0%
	F	Count			1	4	5
		% within Group			20.0%	80.0%	100.0%
Total		Count	6	2	8	4	20
		% within Group	30.0%	10.0%	40.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	23.000[a]	9	.006
Likelihood Ratio	24.953	9	.003
Linear-by-Linear Association	7.300	1	.007
N of Valid Cases	20		

a. 16 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

Group * Connective tissue - Subepithelial - Fibroblasts

Crosstab

			CT - Subep - Fibroblasts		
			+	++	Total
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	16	4	20
		% within Group	80.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.500[a]	3	.475
Likelihood Ratio	3.278	3	.351
Linear-by-Linear Association	.136	1	.713
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Connective tissue - Subepithelial - Inflammatory

Crosstab

			CT - Subep - Inflammatory			
			-	+	++	Total
Group	C	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	S	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	P	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	F	Count		3	2	5
		% within Group		60.0%	40.0%	100.0%
Total		Count	3	13	4	20
		% within Group	15.0%	65.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	11.846[a]	6	.065
Likelihood Ratio	11.990	6	.062
Linear-by-Linear Association	5.347	1	.021
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .75.

Group * Connective tissue - Subepithelial - Hyalinization

Crosstab

			CT - Subep - Hyalinisation		
			Present	Negative	Total
Group	C	Count		5	5
		% within Group		100.0%	100.0%
	S	Count		5	5
		% within Group		100.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	F	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
Total		Count	3	17	20
		% within Group	15.0%	85.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	10.588[a]	3	.014
Likelihood Ratio	10.178	3	.017
Linear-by-Linear Association	7.760	1	.005
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is .75.

Group * Connective tissue - Subepithelial - Vascularity

Crosstab

			CT - Subep - Vascularity		
			+	++	Total
Group	C	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	S	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	P	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	13	7	20
		% within Group	65.0%	35.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.418[a]	3	.490
Likelihood Ratio	2.430	3	.488
Linear-by-Linear Association	1.008	1	.315
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 1.75.

Group * Connective tissue - Deep - Density

Crosstab

			CT - Deep - Dentity			
			+	++	+++	Total
Group	C	Count	1	2	2	5
		% within Group	20.0%	40.0%	40.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	P	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	F	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
Total		Count	9	9	2	20
		% within Group	45.0%	45.0%	10.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	9.333[a]	6	.156
Likelihood Ratio	8.943	6	.177
Linear-by-Linear Association	1.679	1	.195
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

Group * Connective tissue - Deep - Pattern

Crosstab

			CT - Deep - Pattern			
			Wavy	Bundles	Haphazard	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	S	Count	1	1	3	5
		% within Group	20.0%	20.0%	60.0%	100.0%
	P	Count	3		2	5
		% within Group	60.0%		40.0%	100.0%
	F	Count			5	5
		% within Group			100.0%	100.0%
Total		Count	5	4	11	20
		% within Group	25.0%	20.0%	55.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	12.982[a]	6	.043
Likelihood Ratio	14.155	6	.028
Linear-by-Linear Association	2.760	1	.097
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Connective tissue - Deep - Fibroblasts

Crosstab

			CT - Deep - Fibroblasts		
			+	++	Total
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	5		5
		% within Group	100.0%		100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	18	2	20
		% within Group	90.0%	10.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.222[a]	3	.528
Likelihood Ratio	2.995	3	.392
Linear-by-Linear Association	.060	1	.806
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

Group * Connective tissue - Deep - Inflammatory

Crosstab

			CT - Deep - Inflammatory				
			-	+	++	+++	Total
Group	C	Count	3	2			5
		% within Group	60.0%	40.0%			100.0%
	S	Count	1	4			5
		% within Group	20.0%	80.0%			100.0%
	P	Count		1	3	1	5
		% within Group		20.0%	60.0%	20.0%	100.0%
	F	Count		3	2		5
		% within Group		60.0%	40.0%		100.0%
Total		Count	4	10	5	1	20
		% within Group	20.0%	50.0%	25.0%	5.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	16.400[a]	9	.059
Likelihood Ratio	18.626	9	.029
Linear-by-Linear Association	4.778	1	.029
N of Valid Cases	20		

a. 16 cells (100.0%) have expected count less than 5. The minimum expected count is .25.

Group * Connective tissue - Deep - Hyalinization

Crosstab

			CT - Deep - Hyalinisation	
			Negative	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	F	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	20	20
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	20

a. No statistics are computed because CT - Deep - Hyalinisation is a constant.

Group * Connective tissue - Deep - Vascularity

Crosstab

			CT - Deep - Vascularity		
			+	++	Total
Group	C	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	12	8	20
		% within Group	60.0%	40.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	1.667[a]	3	.644
Likelihood Ratio	1.726	3	.631
Linear-by-Linear Association	1.448	1	.229
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 2.00.

Group * Dysplasia

Crosstab

			Dysplatia	
			-	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	F	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	20	20
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	20

a. No statistics are computed because Dysplatia is a constant.

Group * Malignant Islands

Crosstab

			Malignant Islands	
			-	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	F	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	20	20
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Squar	.[a]
N of Valid Cases	20

a. No statistics are computed becaus Malignant Islands is a constant.

Group * Homogenous / Lysis

Crosstab

			Homogenous / Lysis		
			H	L	Total
Group	C	Count	5		5
		% within Group	100.0%		100.0%
	S	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	16	4	20
		% within Group	80.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.500[a]	3	.058
Likelihood Ratio	8.282	3	.041
Linear-by-Linear Association	.000	1	1.000
N of Valid Cases	20		

a. 8 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Vangieson

Crosstab

			Vangieson			
			+	++	+++	Total
Group	C	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
	S	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	P	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
	F	Count		1	4	5
		% within Group		20.0%	80.0%	100.0%
Total		Count	2	13	5	20
		% within Group	10.0%	65.0%	25.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	12.677[a]	6	.048
Likelihood Ratio	14.258	6	.027
Linear-by-Linear Association	6.303	1	.012
N of Valid Cases	20		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

Comparison between Controls, Smokers and Leukoplakia:

Group * Keratinization

Crosstab

			Keratinisation			Total
			O	P	N	
Group	C	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	S	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	L	Count		5		5
		% within Group		100.0%		100.0%
Total		Count	2	12	1	15
		% within Group	13.3%	80.0%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.500[a]	4	.165
Likelihood Ratio	7.097	4	.131
Linear-by-Linear Association	1.114	1	.291
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Thickness

Crosstab

			Thickness	
			H	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	L	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Sc	.[a]
N of Valid Case	15

a. No statistics are computed because Thickness is a co

Group * Interface

Crosstab

			Interface		
			N	T	Total
Group	C	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	L	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	10	5	15
		% within Group	66.7%	33.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squa	.600[a]	2	.741
Likelihood Ratio	.631	2	.729
Linear-by-Linear Association	.500	1	.480
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less tha minimum expected count is 1.67.

Group * Connective tissue - Subepithelial - Density

Crosstab

			CT - Subep - Dentity			
			+	++	+++	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	L	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
Total		Count	7	7	1	15
		% within Group	46.7%	46.7%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.143[a]	4	.273
Likelihood Ratio	5.519	4	.238
Linear-by-Linear Association	3.857	1	.050
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Connective tissue - Subepithelial - Pattern

Crosstab

			CT - Subep - Pattern			
			Wavy	Bundles	Haphazard	Total
Group	C	Count	3	1	1	5
		% within Group	60.0%	20.0%	20.0%	100.0%
	S	Count		1	4	5
		% within Group		20.0%	80.0%	100.0%
	L	Count	1		4	5
		% within Group	20.0%		80.0%	100.0%
Total		Count	4	2	9	15
		% within Group	26.7%	13.3%	60.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.500[a]	4	.165
Likelihood Ratio	8.318	4	.081
Linear-by-Linear Association	2.124	1	.145
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .67.

Group * Connective tissue - Subepithelial - Fibroblasts

Crosstab

			CT - Subep - Fibroblasts		Total
			+	++	
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	L	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	11	4	15
		% within Group	73.3%	26.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig (2-sided)
Pearson Chi-Sq	.682[a]	2	.711
Likelihood Ratio	.659	2	.719
Linear-by-Linear Association	.023	1	.880
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count les minimum expected count is 1.33.

Group * Connective tissue - Subepithelial - Inflammatory

Crosstab

			CT - Subep - Inflammatory				Total
			-	+	++	+++	
Group	C	Count	3	2			5
		% within Group	60.0%	40.0%			100.0%
	S	Count		4	1		5
		% within Group		80.0%	20.0%		100.0%
	L	Count		1		4	5
		% within Group		20.0%		80.0%	100.0%
Total		Count	3	7	1	4	15
		% within Group	20.0%	46.7%	6.7%	26.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	18.000[a]	6	.006
Likelihood Ratio	19.579	6	.003
Linear-by-Linear Association	9.852	1	.002
N of Valid Cases	15		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Connective tissue - Subepithelial - Hyalinization

Crosstab

			CT - Subep - Hyalinisation	Total
			Negative	
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	L	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	15

a. No statistics are computed because CT - Subep - Hyalinisation is a constant.

Group * Connective tissue - Subepithelial - Vascularity

Crosstab

			CT - Subep - Vascularity			
			+	++	+++	Total
Group	C	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	L	Count	2	2	1	5
		% within Group	40.0%	40.0%	20.0%	100.0%
Total		Count	7	7	1	15
		% within Group	46.7%	46.7%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.571[a]	4	.632
Likelihood Ratio	2.747	4	.601
Linear-by-Linear Association	.964	1	.326
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Connective tissue - Deep - Density

Crosstab

			CT - Deep - Dentity			
			+	++	+++	Total
Group	C	Count	1	2	2	5
		% within Group	20.0%	40.0%	40.0%	100.0%
	S	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	L	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
Total		Count	7	6	2	15
		% within Group	46.7%	40.0%	13.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.000[a]	4	.136
Likelihood Ratio	7.442	4	.114
Linear-by-Linear Association	4.400	1	.036
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .67.

Group * Connective tissue - Deep - Pattern

Crosstab

			CT - Deep - Pattern			
			Wavy	Bundles	Haphazard	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	S	Count	1	1	3	5
		% within Group	20.0%	20.0%	60.0%	100.0%
	L	Count	1		4	5
		% within Group	20.0%		80.0%	100.0%
Total		Count	3	4	8	15
		% within Group	20.0%	26.7%	53.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.250[a]	4	.263
Likelihood Ratio	6.279	4	.179
Linear-by-Linear Association	1.207	1	.272
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Connective tissue - Deep - Fibroblasts

Crosstab

			CT - Deep - Fibroblasts		
			+	++	Total
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	S	Count	5		5
		% within Group	100.0%		100.0%
	L	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	14	1	15
		% within Group	93.3%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.143[a]	2	.343
Likelihood Ratio	2.344	2	.310
Linear-by-Linear Association	1.143	1	.285
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Connective tissue - Deep - Inflammatory

Crosstab

			CT - Deep - Inflammatory				
			-	+	++	+++	Total
Group	C	Count	3	2			5
		% within Group	60.0%	40.0%			100.0%
	S	Count	1	4			5
		% within Group	20.0%	80.0%			100.0%
	L	Count		3	1	1	5
		% within Group		60.0%	20.0%	20.0%	100.0%
Total		Count	4	9	1	1	15
		% within Group	26.7%	60.0%	6.7%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	8.167[a]	6	.226
Likelihood Ratio	9.364	6	.154
Linear-by-Linear Association	5.851	1	.016
N of Valid Cases	15		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Connective tissue - Deep - Hyalinization

Crosstab

			CT - Deep - Hyalinisation	
			Negative	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	L	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	15

a. No statistics are computed because CT - Deep - Hyalinisation is a constant.

Group * Connective tissue - Deep - Vascularity

Crosstab

			CT - Deep - Vascularity			
			+	++	+++	Total
Group	C	Count	2	3		5
		% within Grou	40.0%	60.0%		100.0%
	S	Count	3	2		5
		% within Grou	60.0%	40.0%		100.0%
	L	Count	3	1	1	5
		% within Grou	60.0%	20.0%	20.0%	100.0%
Total		Count	8	6	1	15
		% within Grou	53.3%	40.0%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.250[a]	4	.517
Likelihood Ratio	3.506	4	.477
Linear-by-Linear Association	.012	1	.914
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Dysplasia

Crosstab

			Dysplatia		
			+	-	Total
Group	C	Count		5	5
		% within Gr		100.0%	100.0%
	S	Count		5	5
		% within Gr		100.0%	100.0%
	L	Count	5		5
		% within Gr	100.0%		100.0%
Total		Count	5	10	15
		% within Gr	33.3%	66.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Squ	15.000[a]	2	.001
Likelihood Ratio	19.095	2	.000
Linear-by-Linear Association	12.500	1	.000
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less tha minimum expected count is 1.67.

Group * Malignant Islands

Crosstab

			Malignant Islands	
			-	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	S	Count	5	5
		% within Group	100.0%	100.0%
	L	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	15

a. No statistics are computed because Malignant Islands is a constant.

Group * Homogenous / Lysis

Crosstab

			Homogenous / Lysis		
			H	L	Total
Group	C	Count	5		5
		% within Group	100.0%		100.0%
	S	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	L	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
Total		Count	10	5	15
		% within Group	66.7%	33.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	4.200[a]	2	.122
Likelihood Ratio	5.635	2	.060
Linear-by-Linear Association	.980	1	.322
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is 1.67.

Group * Vangieson

Crosstab

			Vangieson			
			+	++	+++	Total
Group	C	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
	S	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	L	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
Total		Count	2	12	1	15
		% within Group	13.3%	80.0%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.000[a]	4	.558
Likelihood Ratio	3.819	4	.431
Linear-by-Linear Association	.091	1	.763
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Comparison between Controls, Pan chewers and Sub mucous fibrosis:

Group * Keratinization

Crosstab

			Keratinisation		
			O	P	Total
Group	C	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	F	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
Total		Count	5	10	15
		% within Group	33.3%	66.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	4.200[a]	2	.122
Likelihood Ratio	5.635	2	.060
Linear-by-Linear Association	.420	1	.517
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is 1.67.

Group * Thickness

Crosstab

			Thickness		
			A	H	Total
Group	C	Count		5	5
		% within Group		100.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	F	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	5	10	15
		% within Group	33.3%	66.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	15.000[a]	2	.001
Likelihood Ratio	19.095	2	.000
Linear-by-Linear Association	10.500	1	.001
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is 1.67.

Group * Interface

Crosstab

			Interface		
			N	T	Total
Group	C	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	F	Count	5		5
		% within Group	100.0%		100.0%
Total		Count	12	3	15
		% within Group	80.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.500[a]	2	.287
Likelihood Ratio	3.278	2	.194
Linear-by-Linear Association	2.333	1	.127
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Connective tissue - Subepithelial - Density

Crosstab

			CT - Subep - Dentity			
			+	++	+++	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	P	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
	F	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
Total		Count	5	9	1	15
		% within Group	33.3%	60.0%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.400[a]	4	.663
Likelihood Ratio	2.634	4	.621
Linear-by-Linear Association	1.135	1	.287
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Connective tissue - Subepithelial - Pattern

Crosstab

			CT - Subep - Pattern				
			Wavy	Bundles	Haphazard	Parallel	Total
Group	C	Count	3	1	1		5
		% within Group	60.0%	20.0%	20.0%		100.0%
	P	Count	3		2		5
		% within Group	60.0%		40.0%		100.0%
	F	Count			1	4	5
		% within Group			20.0%	80.0%	100.0%
Total		Count	6	1	4	4	15
		% within Group	40.0%	6.7%	26.7%	26.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	13.500[a]	6	.036
Likelihood Ratio	16.323	6	.012
Linear-by-Linear Association	7.178	1	.007
N of Valid Cases	15		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Connective tissue - Subepithelial - Fibroblasts

Crosstab

			CT - Subep - Fibroblasts		
			+	++	Total
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	13	2	15
		% within Group	86.7%	13.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	1.154[a]	2	.562
Likelihood Ratio	1.772	2	.412
Linear-by-Linear Association	.000	1	1.000
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is .67.

Group * Connective tissue - Subepithelial - Inflammatory

Crosstab

			CT - Subep - Inflammatory			Total
			-	+	++	
Group	C	Count	3	2		5
		% within Group	60.0%	40.0%		100.0%
	P	Count		4	1	5
		% within Group		80.0%	20.0%	100.0%
	F	Count		3	2	5
		% within Group		60.0%	40.0%	100.0%
Total		Count	3	9	3	15
		% within Group	20.0%	60.0%	20.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	8.667[a]	4	.070
Likelihood Ratio	10.044	4	.040
Linear-by-Linear Association	5.833	1	.016
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Connective tissue - Subepithelial - Hyalinization

Crosstab

			CT - Subep - Hyalinisation		Total
			Present	Negative	
Group	C	Count		5	5
		% within Group		100.0%	100.0%
	P	Count		5	5
		% within Group		100.0%	100.0%
	F	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
Total		Count	3	12	15
		% within Group	20.0%	80.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.500[a]	2	.024
Likelihood Ratio	8.282	2	.016
Linear-by-Linear Association	5.250	1	.022
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Connective tissue - Subepithelial - Vascularity

Crosstab

			CT - Subep - Vascularity		
			+	++	Total
Group	C	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	P	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	11	4	15
		% within Group	73.3%	26.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.682[a]	2	.711
Likelihood Ratio	.659	2	.719
Linear-by-Linear Association	.477	1	.490
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is 1.33.

Group * Connective tissue - Deep - Density

Crosstab

			CT - Deep - Dentity			
			+	++	+++	Total
Group	C	Count	1	2	2	5
		% within Group	20.0%	40.0%	40.0%	100.0%
	P	Count	4	1		5
		% within Group	80.0%	20.0%		100.0%
	F	Count	2	3		5
		% within Group	40.0%	60.0%		100.0%
Total		Count	7	6	2	15
		% within Group	46.7%	40.0%	13.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.000[a]	4	.136
Likelihood Ratio	7.442	4	.114
Linear-by-Linear Association	1.718	1	.190
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .67.

Group * Connective tissue - Deep - Pattern

Crosstab

			CT - Deep - Pattern			
			Wavy	Bundles	Haphazard	Total
Group	C	Count	1	3	1	5
		% within Group	20.0%	60.0%	20.0%	100.0%
	P	Count	3		2	5
		% within Group	60.0%		40.0%	100.0%
	F	Count			5	5
		% within Group			100.0%	100.0%
Total		Count	4	3	8	15
		% within Group	26.7%	20.0%	53.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	12.750[a]	4	.013
Likelihood Ratio	14.056	4	.007
Linear-by-Linear Association	3.201	1	.074
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

Group * Connective tissue - Deep - Fibroblasts

Crosstab

			CT - Deep - Fibroblasts		
			+	++	Total
Group	C	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	13	2	15
		% within Group	86.7%	13.3%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	1.154[a]	2	.562
Likelihood Ratio	1.772	2	.412
Linear-by-Linear Association	.000	1	1.000
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is .67.

Group * Connective tissue - Deep - Inflammatory

Crosstab

			CT - Deep - Inflammatory				
			-	+	++	+++	Total
Group	C	Count	3	2			5
		% within Group	60.0%	40.0%			100.0%
	P	Count		1	3	1	5
		% within Group		20.0%	60.0%	20.0%	100.0%
	F	Count		3	2		5
		% within Group		60.0%	40.0%		100.0%
Total		Count	3	6	5	1	15
		% within Group	20.0%	40.0%	33.3%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	11.800[a]	6	.067
Likelihood Ratio	14.091	6	.029
Linear-by-Linear Association	3.201	1	.074
N of Valid Cases	15		

a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Connective tissue - Deep - Hyalinization

Crosstab

			CT - Deep - Hyalinisation	
			Negative	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	F	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Square	.[a]
N of Valid Cases	15

a. No statistics are computed because CT - Deep - Hyalinisation is a constant.

Group * CT - Deep - Vascularity

Crosstab

			CT - Deep - Vascularity		
			+	++	Total
Group	C	Count	2	3	5
		% within Group	40.0%	60.0%	100.0%
	P	Count	3	2	5
		% within Group	60.0%	40.0%	100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	9	6	15
		% within Group	60.0%	40.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	1.667[a]	2	.435
Likelihood Ratio	1.726	2	.422
Linear-by-Linear Association	1.556	1	.212
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is 2.00.

Group * Dysplasia

Crosstab

			Dysplatia	
			-	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	F	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Squa	.[a]
N of Valid Cases	15

a. No statistics are computed because Dysplatia is a const

Group * Malignant Islands

Crosstab

			Malignant Islands	
			-	Total
Group	C	Count	5	5
		% within Group	100.0%	100.0%
	P	Count	5	5
		% within Group	100.0%	100.0%
	F	Count	5	5
		% within Group	100.0%	100.0%
Total		Count	15	15
		% within Group	100.0%	100.0%

Chi-Square Tests

	Value
Pearson Chi-Squ	.[a]
N of Valid Cases	15

a. No statistics are computed be Malignant Islands is a constan

Group * Homogenous / Lysis

Crosstab

			Homogenous / Lysis		
			H	L	Total
Group	C	Count	5		5
		% within Group	100.0%		100.0%
	P	Count	5		5
		% within Group	100.0%		100.0%
	F	Count	4	1	5
		% within Group	80.0%	20.0%	100.0%
Total		Count	14	1	15
		% within Group	93.3%	6.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.143[a]	2	.343
Likelihood Ratio	2.344	2	.310
Linear-by-Linear Association	1.500	1	.221
N of Valid Cases	15		

a. 6 cells (100.0%) have expected count less than 5. The minimum expected count is .33.

Group * Vangieson

Crosstab

			Vangieson			
			+	++	+++	Total
Group	C	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
	P	Count	1	4		5
		% within Group	20.0%	80.0%		100.0%
	F	Count		1	4	5
		% within Group		20.0%	80.0%	100.0%
Total		Count	2	9	4	15
		% within Group	13.3%	60.0%	26.7%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	11.000[a]	4	.027
Likelihood Ratio	12.816	4	.012
Linear-by-Linear Association	6.105	1	.013
N of Valid Cases	15		

a. 9 cells (100.0%) have expected count less than 5. The minimum expected count is .67.

RESULTS OF AUTOFLUORESCENCE: (AVERAGED EMISSION AND EXCITATION SPECTRUM)

MEAN, STANDARD DEVIATION AND TEST OF SIGNIFICANCE OF MEAN VALUES BETWEEN DIFFERENT GROUPS FOR INTENSITY 380nm AT 320nm EXCITATION

GROUP	In_380 Mean ± SD	P- Value	Significant Groups
I	1490616 ± 1.22		
II	1267554 ± 1.16		
III	179225 ± 1.53	< 0.001(sig.)	I Vs III II Vs III

Table-1: Mean values in group I (1490616 ± 1.22) and group II (1267554 ± 1.16) are significantly higher than the mean value in group III (179225 ± 1.53) with P-Value less than 0.001. However there is no significant difference in mean values between groups I and II (P > O.O5).

MEAN, STANDARD DEVIATION AND TEST OF SIGNIFICANCE OF MEAN VALUES BETWEEN DIFFERENT GROUPS FOR INTENSITY 420nm AT 320nm EXCITATION

GROUP	In_420 Mean ± SD	P- Value	Significant Groups
I	1411410 ± 1.23		
II	1272126 ± 1.19		
III	176398± 1.44	< 0.001(sig.)	I Vs III II Vs III

Table-2: Mean values in group I (1411410 ± 1.23) and group II (1272126 ± 1.19) are significantly higher than the mean value in group III (176398 ± 1.44) with P-Value less than 0.001. However there is no significant difference in mean values between groups I and II (P > 0.005).

MEAN, STANDARD DEVIATION AND TEST OF SIGNIFICANCE OF MEAN VALUES BETWEEN DIFFERENT GROUPS FOR INTENSITY 460nm AT 320nm EXCITATION

GROUP	In_460 Mean ± SD	P- Value	Significant Groups
I	1509969 ± 1.18		
II	1351600 ± 1.22		
III	184444± 1.37	< 0.001(sig.)	I Vs III II Vs III

Table-3: Mean values in group I (1509969 ± 1.23) and group II (1351600 ± 1.22) are significantly higher than the mean value in group III (184444 ± 1.37) with P-Value less than 0.001. However there is no significant difference in mean values between groups I and II (P > 0.005).

RESULTS

RESULTS OF AUTOFLUORESCENCE: (AVERAGED EMISSION AND EXCITATION SPECTRUM)

In the present study, native fluorescence spectroscopy of normal mucosa, mucosa of pan chewers and tobacco smokers were recorded. For the normal individuals and tobacco smokers, readings were taken from the area adjacent to second premolar at occlusal level. In case of pan chewers, readings were taken from mucosa where the quid was placed.

The fluorescence spectra were recorded at these sites, at different wavelengths corresponding to these native fluorophores present in cells and extracellular matrix, such as tryptophan, collagen, elastin and NADH.

Fluorescence emission spectra of oral mucosa at 280nm excitation: (Absorption maximum for tryptophan)

The average emission spectrum of the normal mucosa, mucosa of pan chewers and tobacco smokers, at 280nm excitation corresponding to the amino acid residue, tryptophan molecules are shown in fig 33, with wavelength in the x-axis and fluorescence intensity in Y- axis.

Normal oral mucosa and pan chewer's mucosa showed a prominent peak at 338nm and a small peak at 440nm. It is worth to mention that the emission at 280nm mostly is due to tryptophan, as the emission efficiency (quantum yield) of other two amino acids, phenyl alanine and tyrosine is very minimal as that of tryptophan.

Mucosa of smokers did not show any peaks at 338nm and 440nm.

Normalized emission spectra of oral mucosa at 280nm excitation:

In order to overcome the artefacts of instruments, tissue heterogeneity, normalization of spectra was performed in relation to, either its peak wavelength or any wavelength of interest.

The normalized emission spectra of normal mucosa, mucosa of pan chewers and tobacco smokers at 280nm excitation are shown in fig.34. The normalized spectrum of normal mucosa, mucosa of pan chewers and tobacco smokers showed maximum emission intensity at 340nm wave length.

It is worth noting that the normal tissues showed a lesser intensity than pan chewers and tobacco smokers, at higher wavelengths (400-500nm).

It clearly indicates that NADH/NAD(P)H and collagen distribution may be altered under various tissue transformation conditions.

Fluorescence emission spectra of oral mucosa at 320nm excitation (Absorption spectrum for collagen):

The average emission spectrum of the normal mucosa, mucosa of pan chewers and mucosa of tobacco smokers, at 320nm excitation corresponding to the collagen/elastin are shown in fig 35.

The emission spectra corresponding to normal mucosa and pan chewer's mucosa showed two prominent peaks at 390nm and 460nm respectively. The first peak which is at 390nm corresponds to the collagen intensity (around 390) and the second peak corresponds to the NADH (around 460nm), which are almost equal.

However, surprisingly the mucosa of tobacco smokers did not show any increase in intensity at 390nm which corresponds to the amount of collagen.

The second peak at 460nm which corresponds to NADH in the tissues was also not seen in tobacco smokers.

Normalized emission spectra of oral mucosa at 320nm excitation:

The normalized emission spectra of normal mucosa, mucosa of pan chewers and tobacco smoker's mucosa at 320nm excitation are shown in fig.36. All the three spectra i.e., normal patients, pan chewers and tobacco smokers showed maximum emission at 390nm wavelength. A second peak of increased wavelength is observed at 451nm for normal, 452nm for pan chewers and 439nm for tobacco smokers was observed.

Fluorescence excitation (absorption) spectra of oral mucosa at 340nm emission:

The average excitation spectra of the normal mucosa, mucosa of pan chewers and mucosa of tobacco smokers, at 340nm emission corresponding to the tryptophan are shown in fig 37.

The emission spectra corresponding to normal mucosa showed prominent intensity at 295nm whereas the pan chewer's mucosa showed a prominent peak at 296nm. Tobacco smoker's mucosa showed a small peak, with maximum intensity at 292nm wavelength. This picture shows that the maximum intensity is shown by the normal individuals followed by pan chewers which showed a slightly decreased intensity compared to the normal individuals.

Normalized excitation spectra of oral mucosa at 340nm emission:

The normalized excitation spectra of normal mucosa, mucosa of pan chewers and mucosa of tobacco smokers at 340nm emission are shown in fig.38.

Pan chewers did not show significant peak shift compared to the normal individuals, both of them is showing maximum intensity at 295nm. Surprisingly, patients who are tobacco smokers showed maximum intensity at 292nm showing a shift to the left i.e., towards shorter wavelength. This shift to the left could probably attributed to any conformational changes in the tissue protein sequences and/or partial folding of protein structures.

Fluorescence excitation spectra of oral mucosa at 390nm emission:

The average excitation spectra of the normal mucosa, mucosa of pan chewers and mucosa of tobacco smokers, at 390nm excitation corresponding to the collagen are shown in fig 39.

The excitation spectra corresponding to normal mucosa and pan chewers mucosa showed relatively a prominent peak at 295nm, where as tobacco smokers showed a small peak at the same wavelength. However, it is worth to note that the excitation intensity of pan chewers is much less compared to normal at this emission spectrum.

Normalized excitation spectra of oral mucosa at 390nm emission:

The normalized emission spectra of normal mucosa, mucosa of pan chewers and tobacco smokers at 390nm emission are shown in fig.40. The emission spectral findings at 390nm are similar to that of 340nm emission. However a slight shift to the left (towards shorter wavelength) is seen in case of

pan chewers when compared to the normal individuals. Patients who are tobacco smokers showed a gross shift to the left as seen in 340nm emission. The maximum intensity values at this emission spectrum for normal, pan chewers and oral submucous fibrosis are 298, 297 and 294 respectively. This clearly indicates that the absorption bands of collagen vary with tissue conditions.

DISCRIMINATION OF TOBACCO SMOKERS FROM NORMAL INDIVIDUALS:

As there is considerable difference in the autofluorescence spectra between healthy and tobacco smokers, an attempt is also made to verify the diagnostic potentiality of this technique. We also attempted to discriminate the betel quid chewers from normal patients and tobacco smokers from betel quid chewers.

In this context, different intensity values for excitation 320nm were introduced. Among various excitation wavelengths, the intensity values at 380, 420 and 460 at 320nm excitation were selected for subsequent analysis, as they showed considerable differences between normal and tobacco smokers.

DISCRIMINATION USING PEAK INTENSITY VALUE:

Figure 41 shows the fluorescence intensity at 380nm for normal and pan chewers at 320nm excitation. From the figure, it is observed that there is considerable overlap in the fluorescence intensity at I-380 between normal and pan chewers. It is also observed that this intensity value discriminates normal from betel quid chewers with a sensitivity of 78% and specificity of 12%.

Figure 42 shows the fluorescence intensity at 380nm for pan chewers and tobacco smokers at 320nm excitation. From the figure, it is observed that

there is considerable difference in the fluorescence intensity at I-380 between pan chewers and tobacco smokers. It is also observed that this intensity value discriminates normal from tobacco smokers with a sensitivity of 100% and specificity of 100%.

Figure 43 shows the fluorescence intensity at 380nm for pan chewers and tobacco smokers at 320nm excitation. From the figure, it is observed that there is considerable difference in the fluorescence intensity at I-380 between pan chewers and tobacco smokers. From the figure, it is observed that this intensity value discriminates tobacco smokers from pan chewers with a sensitivity of 100% and specificity of 100%.

The Mean, Standard deviation and test of significance of mean values between different groups for intensity-380 nm at 320 nm excitation *shows that the Mean values in group I (1490616* ± *1.22) and group II (1267554* ± *1.16) are significantly higher than the mean value in group III (179225* ± *1.53) with a p-value of less than 0.001. However there is no significant difference in mean values between groups I and II (P >0.05) (**Table-1**).*

The Mean, Standard deviation and test of significance of mean values between different groups for intensity-420 nm at 320 nm excitation *shows that the mean values in group I (1411410* ± *1.23) and group II (1272126* ± *1.19) are significantly higher than the mean value in group III (176398* ± *1.44) with a p-value of less than 0.001. However there is no significant difference in mean values between groups I and II (P > 0.05).* ***(Table-2)***

The Mean, Standard deviation and test of significance of mean values between different groups for intensity-460 nm at 320 nm excitation *shows that the Mean values in group I (1509969 ± 1.23) and group II (1351600 ± 1.22) are significantly higher than the mean value in group III (184444 ± 1.37) with a p-value of less than 0.001. However there is no significant difference in mean values between groups I and II (P > 0.005).* ***(Table-3****)*

RESULTS OF HISTOPATHOLOGICAL ASSESSMENT:

Histopathological assessment was performed and the following combinations were assessed for statistical significance,

1. *Comparison among the pan chewers, smokers and non-users of tobacco.*
2. *Comparison among the pan chewers, smokers, non-users of tobacco, leukoplakia, sub mucous fibrosis, well differentiated squamous cell carcinoma, moderately differentiated squamous cell carcinoma and verrucous carcinoma.*
3. *Comparison among the pan chewers, smokers, non-users of tobacco, leukoplakia and sub mucous fibrosis.*
4. *Comparison among the smokers, non-users of tobacco and leukoplakia.*
5. *Comparison among the pan chewers, non-users of tobacco and sub mucous fibrosis.*

Comparison among the pan chewers, smokers and non-users of tobacco:

1. ***Presence of inflammatory cells in the deeper region of the connective tissue (p=0.036) was significant.*** *Among the non-users of tobacco, 3 cases did not have any inflammatory cells and 2 cases had mild inflammatory infiltrate. Among the smokers, 1 case did not have any inflammatory infiltrate and 4 cases had mild inflammatory infiltrate. Among the pan chewers, 1 case had mild inflammatory infiltrate, 3 cases had moderate inflammatory infiltrate and 1 case had severe inflammatory infiltrate (fig 14).*
2. ***Presence of lysis in the connective tissue (p=0.024) was significant.*** *All the 5 cases of non-users of tobacco and 5 cases of pan chewers did not show lysis in the connective tissue. Among smokers, 3 cases showed lysis and 2 cases did not show any lysis (fig 15).*
3. *The other parameters of keratinization of the epithelium, thickness of the epithelium, interface between the epithelium and connective tissue, density of connective tissue, pattern of connective tissue, number of fibroblasts, presence of inflammatory cells, hyalinization and amount of vascularity in the sub-epithelial region, density of connective tissue, pattern of connective tissue, number of fibroblasts, hyalinization and amount of vascularity in the deeper region, presence of dysplasia, malignant islands and intensity of van Gieson stain did not achieve statistical significance.*

Comparison among the pan chewers, smokers, non-users of tobacco, leukoplakia, sub mucous fibrosis, well differentiated squamous cell carcinoma, moderately differentiated squamous cell carcinoma and verrucous carcinoma:

1. ***The nature of the keratinized epithelium (p = o.o34) was significant****. Among non-users of tobacco, 2 cases had ortho-keratinized epithelium and 3 cases had para-keratinized epithelium. Among smokers, 4 cases had para-keratinized epithelium and 1 case had non-keratinized epithelium. Among sub mucous fibrosis, 3 cases had ortho-keratinized epithelium and 2 cases had para-keratinized epithelium. All the remaining groups had para-keratinized epithelium (fig 2).*
2. ***The thickness of epithelium (p=0.000) was highly significant.*** *All cases of sub-mucous fibrosis had atrophied epithelium. All the remaining groups had hyperplastic epithelium.*
3. ***The nature of the interface between epithelium and connective tissue (p= 0.000) was highly significant.*** *Among non users of tobacco and smokers, the interface was normal in 3 cases and thickened in 2 cases. Among pan chewers and leukoplakia, the interface was normal in 4 cases and thickened in one case. The interface was discontinuous in all the cases of well differentiated and moderately differentiated carcinoma. The interface was normal in all cases of verrucous carcinoma (fig 3).*
4. ***The pattern of sub-epithelial connective (p=0.000) was highly significant.*** *Among non-users of tobacco, 3 cases had wavy pattern, 1*

case had bundles and 1 case had haphazard arrangement. Among smokers, 1 case had bundles and 4 cases had haphazard arrangement. Among pan chewers, 3 cases had wavy pattern and 2 cases had haphazard arrangement. Among leukoplakia, 1 case had wavy pattern and 4 cases had haphazard arrangement. Among sub mucous fibrosis, the pattern was haphazard in 1 case and parallel in 4 cases. Among well differentiated squamous cell carcinoma, 1 case had wavy pattern and 4 cases had haphazard arrangement. Among moderately differentiated squamous cell carcinoma, 3 cases had haphazard arrangement and 2 cases had a stream pattern. Among verrucous carcinoma, all the 5 cases had haphazard arrangement (fig 4).

5. ***Presence of sub-epithelial inflammatory cells (p=0.000) is highly significant.*** *Among the non-users of tobacco, 3 cases did not have any inflammatory cells and 2 cases had mild inflammatory infiltrate. Among the smokers and pan chewers, 1 case had moderate inflammatory infiltrate and 4 cases had mild inflammatory infiltrate. Among leukoplakia, 1 case had mild inflammatory cells and 4 cases had severe inflammatory infiltrate. Among sub-mucous fibrosis, 2 cases had moderate inflammatory infiltrate and 3 cases had mild inflammatory infiltrate. Among well differentiated squamous cell carcinoma, 1 case had mild inflammatory infiltrate and 4 cases had severe inflammatory infiltrate. Among moderately differentiated squamous cell carcinoma, 1 case had mild inflammation, 3 cases had moderate and 1 case had severe*

inflammatory infiltrate. Among verrucous carcinoma, 1 case had moderate and 4 cases had severe inflammatory infiltrate (fig 5).

6. ***Presence of sub-epithelial hyalinization (0.002) was very significant.*** *Among sub mucous fibrosis, 3 cases had hyalinization and 2 cases did not have hyalinization. All the other groups did not have hyalinization (fig 6).*
7. ***The connective tissue pattern in the deeper region (p=0.004) was very significant.*** *Among non-users of tobacco, 1 case had wavy pattern, 3 cases had bundles and 1 case had haphazard arrangement. Among smokers, 1 case had bundles, 1 case had wavy pattern and 3 cases had haphazard arrangement. Among pan chewers, 3 cases had wavy pattern and 2 cases had haphazard arrangement. Among leukoplakia, 1 case had wavy pattern and 4 cases had haphazard arrangement. Among sub mucous fibrosis, the pattern was haphazard in 5 cases. Among well differentiated squamous cell carcinoma, 1 case had wavy pattern and 4 cases had haphazard arrangement. Among moderately differentiated squamous cell carcinoma, 3 cases had haphazard arrangement and 2 cases had a stream pattern. Among verrucous carcinoma, all the 5 cases had haphazard arrangement (fig 7).*
8. ***The number of fibroblasts in the deeper region (p=0.016) was very significant.*** *Among non-users of tobacco and sub mucous fibrosis, 4 cases had minimal and 1 case had moderate amount of fibroblasts. Among smokers, pan chewers and leukoplakia, 5 cases had minimal fibroblasts. Among well differentiated carcinoma, 1 case had minimal*

and 4 cases had moderate amount of fibroblasts. In moderately differentiated carcinoma and verrucous carcinoma, 2 cases had minimal and 3 cases had moderate number of fibroblasts (fig 8).

9. ***The number of inflammatory cells in the deeper region (p=0.002) is highly significant.*** *Among the non-users of tobacco, 3 cases did not have any inflammatory cells and 2 cases had mild inflammatory infiltrate. Among the smokers, 1 case did not have any inflammatory cells and 4 cases had mild inflammatory infiltrate. Among pan chewers, 3 cases had moderate inflammatory infiltrate, 1 case had severe inflammatory infiltrate and 1 case had mild inflammatory infiltrate. Among leukoplakia, 3 cases had mild inflammatory cells, 1 case had moderate and 1 case had severe inflammatory infiltrate. Among sub-mucous fibrosis, 2 cases had moderate inflammatory infiltrate and 3 cases had mild inflammatory infiltrate. Among well differentiated squamous cell carcinoma, 1 case had mild inflammatory infiltrate and 4 cases had severe inflammatory infiltrate. Among moderately differentiated squamous cell carcinoma, 4 cases had moderate and 1 case had severe inflammatory infiltrate. Among verrucous carcinoma, 1 case had mild, 2 cases had moderate and 2 cases had severe inflammatory infiltrate (fig 9).*

10. ***Presence of epithelial dysplasia (p=0.000) was highly significant.*** *Dysplasia was not evident among non-users, smokers, pan chewers and sub mucous fibrosis. Dysplasia was evident among*

leukoplakia, well differentiated and moderately differentiated carcinoma and verrucous carcinoma (fig 10).

11. ***Presence of malignant islands in connective tissue (p=0.000) was highly significant.*** *Malignant islands were absent in non-users, smokers, pan chewers, leukoplakia, sub mucous fibrosis and verrucous carcinoma. Malignant islands were present in well differentiated and moderately differentiated carcinomas (fig 11).*
12. ***The presence of lysis in the connective tissue (p=0.05) was significant.*** *All the 5 cases of non-users of tobacco and 5 cases of pan chewers did not show lysis in the connective tissue. Among smokers, 3 cases showed lysis and 2 cases did not show any lysis. Among leukoplakia, 2 cases showed lysis and 3 cases did not show lysis. Among sub mucous fibrosis, 1 case showed lysis and 4 cases did not show lysis. Among well differentiated carcinoma, 4 cases showed lysis and 1 case did not show lysis. Among moderately differentiated carcinoma, 3 cases showed lysis and 2 cases did not show lysis. Among verrucous carcinoma, 4 cases did not show any lysis and 1 case showed lysis (fig 12).*
13. ***The intensity of van Gieson stain (p=0.002) was highly significant.*** *Among non-users, pan chewers and leukoplakia, 1 case had minimal intensity and 4 cases had moderate intensity. Among smokers, 4 cases had moderate intensity and 1 case had increased intensity. Among sub mucous fibrosis, 1 case had moderate intensity and 4 cases had increased intensity. Among well differentiated carcinoma, 2 cases had*

minimal intensity and 3 cases had moderate intensity. Among moderately differentiated carcinoma, 4 cases had minimal intensity and 1 case had moderate intensity. All the 5 cases of verrucous carcinoma had moderate intensity (fig 13).

14. *The other parameters of density of connective tissue, number of fibroblasts and amount of vascularity in the sub-epithelial region, density of connective tissue, presence of hyalinization and amount of vascularity in the deeper region did not achieve statistical significance.*

Comparison among the pan chewers, smokers, non-users of tobacco and leukoplakia:

1. ***Presence of inflammatory cells in the sub-epithelial region (p=0.003) was highly significant.*** *Among the non-users of tobacco, 3 cases did not have any inflammatory cells and 2 cases had mild inflammatory infiltrate. Among the smokers and pan chewers, 1 case had moderate inflammatory infiltrate and 4 cases had mild inflammatory infiltrate. Among leukoplakia, 1 case had mild inflammatory cells and 4 cases had severe inflammatory infiltrate (fig 25).*
2. ***Presence of epithelial dysplasia (p=0.000) was highly significant.*** *Dysplasia was not evident among non-users, smokers and pan chewers. Dysplasia was evident among leukoplakia (fig 26).*
3. *The other parameters of keratinization of the epithelium, thickness of the epithelium, interface between the epithelium and connective tissue, density of connective tissue, pattern of connective tissue, number of fibroblasts, hyalinization and amount of vascularity in the sub-epithelial*

region, density of connective tissue, pattern of connective tissue, presence of inflammatory cells, number of fibroblasts, hyalinization and amount of vascularity in the deeper region, malignant islands, status of connective tissue and intensity of van Gieson stain did not achieve statistical significance.

Comparison among the pan chewers, smokers, non-users of tobacco, and sub mucous fibrosis:

1. ***The thickness of the epithelium (p=0.000) was highly significant.*** *All cases of sub-mucous fibrosis had atrophied epithelium. All the remaining groups had hyperplastic epithelium (fig 27).*
2. ***The pattern of connective tissue in the sub-epithelial region (p=0.006) was highly significant.*** *Among non-users of tobacco, 3 cases had wavy pattern, 1 case had bundles and 1 case had haphazard arrangement. Among smokers, 1 case had bundles and 4 cases had haphazard arrangement. Among pan chewers, 3 cases had wavy pattern and 2 cases had haphazard arrangement. Among sub mucous fibrosis, the pattern was haphazard in 1 case and parallel in 4 cases (fig 28).*
3. ***Presence of hyalinization in the sub-epithelial connective tissue (p=0.014) was very significant.*** *Among sub mucous fibrosis, 3 cases had hyalinization and 2 cases did not have hyalinization. All the other groups did not have hyalinization (fig 44).*
4. ***The pattern of connective tissue in the deeper region (p=0.043) was significant.*** *Among non-users of tobacco, 1 case had wavy pattern, 3 cases had bundles and 1 case had haphazard arrangement.*

Among smokers, 1 case had bundles, 1 case had wavy pattern and 3 cases had haphazard arrangement. Among pan chewers, 3 cases had wavy pattern and 2 cases had haphazard arrangement. Among sub mucous fibrosis, the pattern was haphazard in 5 cases (fig 29).

5. ***Presence of inflammatory cells in the deeper region (p=0.05) was significant.*** *Among the non-users of tobacco, 3 cases did not have any inflammatory cells and 2 cases had mild inflammatory infiltrate. Among the smokers, 1 case did not have any inflammatory cells and 4 cases had mild inflammatory infiltrate. Among pan chewers, 3 cases had moderate inflammatory infiltrate, 1 case had severe inflammatory infiltrate and 1 case had mild inflammatory infiltrate. Among sub-mucous fibrosis, 2 cases had moderate inflammatory infiltrate and 3 cases had mild inflammatory infiltrate (fig 30).*
6. ***Presence of lysis (p-0.005) was significant.*** *All the 5 cases of non-users of tobacco and 5 cases of pan chewers did not show lysis in the connective tissue. Among smokers, 3 cases showed lysis and 2 cases did not show any lysis. Among sub mucous fibrosis, 1 case showed lysis and 4 cases did not show lysis (fig 31).*
7. ***The intensity of van Gieson stain (p=0.048) was significant.*** *Among non-users and pan chewers, 1 case had minimal intensity and 4 cases had moderate intensity. Among smokers, 4 cases had moderate intensity and 1 case had increased intensity. Among sub mucous fibrosis, 1 case had moderate intensity and 4 cases had increased intensity (fig 32).*

8. *The other parameters of keratinization of the epithelium, interface between the epithelium and connective tissue, density of connective tissue, number of fibroblasts, number of inflammatory cells and amount of vascularity in the sub-epithelial region, density of connective tissue, number of fibroblasts, hyalinization and amount of vascularity in the deeper region, epithelial dysplasia and malignant islands did not achieve statistical significance.*

Comparison among the smokers, non-users of tobacco and leukoplakia:

1. ***Presence of sub-epithelial inflammatory cells (p=0.006) was highly significant.*** *Among the non-users of tobacco, 3 cases did not have any inflammatory cells and 2 cases had mild inflammatory infiltrate. Among the smokers, 1 case had moderate inflammatory infiltrate and 4 cases had mild inflammatory infiltrate. Among leukoplakia, 1 case had mild inflammatory infiltrate and 4 cases had severe inflammatory infiltrate (fig 18).*
2. ***Presence of epithelial dysplasia (p=0.001) was highly significant.*** *Dysplasia was not evident among non-users and smokers. Dysplasia was evident among leukoplakia (fig 19).*
3. *The other parameters of keratinization of the epithelium, thickness of the epithelium, interface between the epithelium and connective tissue, density of connective tissue, pattern of connective tissue, number of fibroblasts, hyalinization and amount of vascularity in the sub-epithelial region, density of connective tissue, pattern of connective tissue, number*

of fibroblasts, presence of inflammatory cells, hyalinization and amount of vascularity in the deeper region, malignant islands, presence of lysis and intensity of van Gieson staining did not achieve statistical significance.

Comparison among the pan chewers, non-users of tobacco and sub mucous fibrosis:

1. ***The thickness of epithelium (p=0.001) was highly significant.*** *All cases of sub-mucous fibrosis had atrophied epithelium. All the remaining groups had hyperplastic epithelium (fig 20).*
2. ***The pattern of connective tissue in the sub-epithelial region (p=0.036) was significant.*** *Among non-users of tobacco, 3 cases had wavy pattern, 1 case had bundles and 1 case had haphazard arrangement. Among pan chewers, 3 cases had wavy pattern and 2 cases had haphazard arrangement. Among sub mucous fibrosis, the pattern was haphazard in 1 case and parallel in 4 cases (fig 21).*
3. ***Presence of hyalinization in the sub-epithelial connective tissue (p=0.024) was significant.*** *Among sub mucous fibrosis, 3 cases had hyalinization and 2 cases did not have hyalinization. All the other groups did not have hyalinization (fig 22).*
4. ***The pattern of connective tissue in the deeper region (p=0.013) was very significant.*** *Among non-users of tobacco, 1 case had wavy pattern, 3 cases had bundles and 1 case had haphazard arrangement. Among pan chewers, 3 cases had wavy pattern and 2 cases had*

haphazard arrangement. Among sub mucous fibrosis, the pattern was haphazard in 5 cases (fig 23).

5. ***The intensity of van Gieson stain (p=0.027) was significant.*** *Among non-users and pan chewers, 1 case had minimal intensity and 4 cases had moderate intensity. Among sub mucous fibrosis, 1 case had moderate intensity and 4 cases had increased intensity (fig 24).*
6. *The other parameters of keratinization of the epithelium, interface between the epithelium and connective tissue, density of connective tissue, number of fibroblasts, number of inflammatory cells and amount of vascularity in the sub-epithelial region, density of connective tissue, number of fibroblasts, number of inflammatory cells, hyalinization and amount of vascularity in the deeper region, epithelial dysplasia, presence of lysis and malignant islands did not achieve statistical significance.*

Findings of Transmission electron microscopy:

The findings of TEM study of the samples are as follows,

Controls:

Presence of continuous, homogeneous, thick collagen bundles with occasional blood vessels and numerous fibroblasts. The fibroblasts are spindle / elongated in shape with elongated nucleus, dense chromatin, numerous endoplasmic reticulum and extended cisternae.

Smokers:

Presence of numerous discontinuous bundles, few dense granules, There are few fibroblasts actively secreting collagen. There are increased bundles of collagen. The fibroblasts are elongated to spindle shaped, with elongated

nucleus pushed to one end, numerous endoplasmic reticulum, extended cisternae, granules filled with proteinaceous material. There is presence of mast cells. The collagen bundles show prominent banding.

Pan chewers:

Presence of immature, thin filaments and the banding was not prominent. The fibroblasts are epithelioid in shape with a round nucleus, prominent nucleolus and endoplasmic reticulum. There is evidence of increased cellularity. There is presence of many keratinocytes and few melanocytes with melanin granules in the connective tissue. There are highly vacuolated cells with vacuoles containing some debris. There is presence of degenerating cells or apoptotic cells with damaged organelles, fragmented organelles and apoptotic bodies. Presence of few lymphoid cells predominantly granulocytes.

Increase in fibers, haphazard arrangement of fibrils and reduced cellularity. There is presence of fat cells or fat accumulation with fat globules. The fibroblasts are stellate in shape. There is presence of degenerating cells and numerous vacant spaces.

GRAPHS

DISCUSSION

Discussion

Tobacco is used in many ways. The most basic difference being between those in which tobacco is smoked and those in which unburnt tobacco is maintained in contact with the oral mucosa. The relative risk of oral cancer is increased between 10 – 15 fold in those who chew tobacco [27].

It has been estimated that cancers in tobacco and alcohol users develop about 15 years earlier than in people who neither smoke nor drink. Much emphasis has been given to precancer and cancer throughout the literature. The role of connective tissue has not been given much attention in comparison with studies on the epithelium.

Thus we designed the study to evaluate different parameters with a special emphasis on connective tissue using three different tools namely, Autofluorescence, Histopathology and Transmission Electron microscopy.

We therein selected patients who are pan chewers or smokers for the past 5 years, who however did not have any visible changes in the buccal mucosa for the study along with controls.

The study comprised of 30 subjects, 25 males and 5 females aged between 25 – 40 years. The control group, who did not use tobacco, comprised of 5 males and 5 females. The study group of pan chewers comprised of 10 males who use atleast 5 packets a day, for a minimum period of 1 year to a maximum of 5 years. They predominantly used branded commercial tobacco products. The study group of smokers comprised of 10 males who use atleast 5 cigarettes a

day for a minimum period of 1year to a maximum of 5 years. They predominantly used filter cigarettes.

The study also compared only the histological changes of the study group with leukoplakia, oral sub mucous fibrosis, well differentiated squamous cell carcinoma, moderately differentiated squamous cell carcinoma and verrucous carcinoma.

The criteria for the assessment were based on the physical state of collagen, overall fibroblastic response and inflammatory response.

Autofluorescence was the first tool to be used for investigation so as to get the maximum representation, if at all there is any change in the connective tissue. Autofluorescence is a non-invasive, easily applicable tool for the detection of alterations in the structural and chemical composition of cells, which may include the presence of diseased tissue. Autofluorescence of tissues is produced by several endogenous fluorophores and it comprises of fluorophores from the tissue matrix like NADH and FAD. Autofluorescence spectroscopy is also useful in guiding the clinician to the optimal location for biopsy and has shown promising results for screening purposes.

The present study is aimed at identifying the mucosal changes on regular usage of pan and smoking. The tissue fluorescence (Emission and Excitation spectra) were recorded for different fluorophores present in the oral mucosa. Because of the inherent fluorescent property of the native fluorophores, tissue fluorophores emit fluorescence when excited at particular wavelength. This

emission of fluorescence by tissues varies for each molecule, according to the wavelength used to excite the tissue.

Tryptophan shows maximum intensity at 280nm, collagen at 320nm, NADH at 340nm and endogenous porphyrin at 405nm excitation. Accordingly, autofluorescence spectra were performed corresponding to these wavelengths for normal mucosa and mucosa of pan chewers and smokers.

Chen et al [194] performed invivo autofluorescence spectra at 330nm excitation. They showed increased fluorescence intensity at 380nm which corresponds to wavelength of collagen, in sub mucous fibrosis when compared with controls.

Tsai et al [195] reported decreased intensity at 380nm and attributed this to distortion of fluorescence emission caused by collagen in sub mucous fibrosis.

The fluorescence characteristics in the present study were analyzed based on two procedures, which are

- *Fluorescence emission spectroscopy*
- *Fluorescence excitation spectroscopy*

Analysis using Fluorescence emission spectroscopy:

With fluorescence emission spectroscopy, we can obtain the exact wavelength at which there is maximum fluorescence intensity for each natural fluorophores. This technique detects the amount of natural fluorophores present in the tissue. So, based on the changes in the spectra of normal fluorophores, we can identify the disease process.

In the present study, the averaged fluorescence emission spectrum of normal mucosa, mucosa of pan chewers and smokers, at 280nm and 320nm excitation wavelengths were performed.

The averaged fluorescence emission spectra of normal mucosa, mucosa of pan chewers and smokers at 280nm corresponding to the tryptophan molecules. The salient features are,

- *Normal oral mucosa and mucosa of pan chewers showed a prominent peak at 338nm and a small secondary peak at 440nm.*
- *Mucosa of smokers showed a small peak at 338nm without a secondary peak at 440nm.*
- *The normalized emission spectra of normal mucosa, mucosa of pan chewers and smokers at 280nm are same with the maximum intensity at 338nm.*

Although the normalized spectral signature of smokers is similar to that of pan chewers and normal mucosa, the absolute intensity is 8 folds lesser than that of normal mucosa. The major peak around 338nm may be attributed to tryptophan emission.

It is confirmed that the tissues at 280nm excitation emit similar wavelengths; however the relative distribution of tryptophan molecules and/or their sequence is different from histological conditions of the tissue. This decrease in tryptophan levels may be attributed to the sparse Cellularity or it may also be due to the distortion of fluorescent intensity in smokers.

The autofluorescence emission spectra of normal mucosa, mucosa of pan chewers and smokers at 320nm excitation corresponding to collagen was performed and the salient features are,

- *First peak around 390nm corresponds to emission from collagen and elastin*
- *Second peak at 460nm corresponds to NADH present in epithelial cells of the surface mucosa*
- *Valley between the two peaks corresponds to the hemoglobin absorption of the tissues.*
- *The emission spectra of normal mucosa and pan chewers showed two prominent peaks centered at 390nm and 460nm with a valley around 420nm. However the relative intensity of emission of pan chewers is slightly lower than that of normal individuals.*
- *The mucosa of smokers has only one broad peak in the region of 370nm to 530nm without any dip at 420nm indicating less vascularity*

The peak at 390nm corresponds to fluorescence intensity by the collagen and elastin present in the tissues. It is surprising to observe that smokers displayed decreased fluorescent intensity compared to that of normal mucosa and pan chewers at this particular wavelength. This observation is contradictory to that of Chen et al [194] who found increased collagen intensity in sub mucous fibrosis. However, our results are similar to findings of Tsai et al [195]

who found a decreased intensity in sub mucous fibrosis when compared with controls.

The second peak at 440nm corresponds to fluorescence emission of NADH present in the epithelial cells. The changes in epithelium may result in increased NADH intensity. However, the NADH intensity in pan chewers is slightly less compared to that of normal. This might be due to minimal scattering caused by collagen that is beginning to accumulate in the submucosa of these individuals. In case of smokers, no peak was observed at 440nm, which probably could be attributed to the distortion of fluorescence caused by collagen.

The valley that is observed around 420nm corresponds to hemoglobin absorption. The valley indicates the amount of vascularity in the tissues. Normal mucosa and pan chewers showed a prominent dip around 420nm. But smokers did not show any dip at this wavelength. It is also interesting to note that spectral signature of smokers is entirely different from normal and pan chewers. The spectral band of normal and pan chewers show a valley around 420nm and 580nm and this indicates a considerable vascularity in these tissues. However the valley at 420nm and 580nm are completely absent in smokers indicating minimal vascularity.

Analysis using fluorescent excitation spectra:

This is a complimentary technique which is sensitive to any conformational changes that take place during the process of tissue transformation. It will provide the changes in absorption band of molecules.

Inorder to get the emission spectrum, the molecule of interest should be excited at its exact absorption wavelength.

Generally it is very difficult to measure absorption spectra of turbid media like cells and tissues. Under such conditions, fluorescence excitation spectra can indirectly measure the absorption band. The peak emission of the fluorophores is monitored at different excitation wavelengths. The maximum intensity corresponding to a particular wavelength of excitation is the absorption wavelength of the molecule. Hence, we also monitored the fluorescence excitation spectra for all the tissues, for their emission at 340nm and 390 nm to confirm that the emission at 340nm is due to tryptophan and to check whether there is any change in the absorption band, when normal tissues undergo various transformations.

A shift to the right, i.e. to higher wavelength is seen in case of malignancy and is called Red shift. Similarly a shift to the left is also observed in some situations and is called Left shift or Blue shift.

It is found that normal and pan chewers have similar absorption bands at 300nm. However smokers have very minimal absorption (30 times less than normal). This may be due to the scattering or reflection of light due to decreased blood supply. Though there are many controversies behind the decrease in fluorescence, our results correlate with Tsai et al [195] and he attributed the decrease in fluorescence in sub mucous fibrosis to the distortion of fluorescence caused by excess collagen in the sub mucosa.

The average excitation spectra of normal, mucosa of pan chewers and smokers at 340nm emission corresponding to tryptophan reveals,

- *The emission spectra corresponding to normal mucosa showed prominent intensity at 295nm*
- *Pan chewers mucosa showed a prominent peak at 296nm*
- *Smokers mucosa showed a small peak at 292nm*
- *In normalized spectra at 340nm, smokers showed a shift to the left when compared to the normal.*

The results show the maximum intensity of normal mucosa, followed by pan chewers who showed a slightly decreased intensity compared to normal and smokers showed the minimum absorption.

The normalized spectra of smokers show the left shift compared to normal indicating that there is a considerable rearrangement in the protein sequences of amino acids.

Liang explained this left shift by photophysical characteristics of tryptophan which depends on the micro-environmental conditions. In particular, the emission of tryptophan depends upon its solvent polarity. The shift to shorter wavelength occurs as the solvents surrounding the tryptophan residues decrease. This explanation could be applied to smokers, as there is stabilization of collagen, making them less soluble to collagenases when compared to normal collagen. Another explanation for the left shift or the decrease in tryptophan intensity could be due to the sparse Cellularity in the

dense collagenized sub mucosa. Another reason for the decrease in collagen fluorescence in spite of having more amounts of collagen and elastin is that there may be a reflection of light at the epithelium-connective tissue interface without any absorption. This may be due to the change in the refractive index of the tissues.

In order to improve the diagnostic interpretation, different parameters were introduced at the emission peak of 320nm excitation. We selected 320nm excitation because the presence of fluorophores – collagen, hemoglobin and NADH that are altered in smokers. The wavelengths were selected at points where maximum intensity of corresponding fluorophores was observed. Based on these intensity values, we performed statistical analysis.

At 320nm excitation, the intensity values at 380nm, 420nm and 460nm were selected. They showed significant statistical difference between normal and smokers, pan chewers and smokers with p value of less than 0.001. However the difference between the normal and pan chewers with p value of greater than 0.005.

The mean intensity was calculated for the study groups. The cut off value for normal and smokers had significant difference and is around the intensity of 700000. The cut off for pan chewers and smokers is also around 700000 with a sensitivity and specificity of 100%. The cut off value of 1159216 was detected with a sensitivity of 77% and specificity of 11%.

The intensity values at 420nm followed a similar pattern to 380nm values described above. Smokers were differentiated from normal and pan

chewers with a sensitivity and specificity of 100%. Normal and pan chewers showed considerable overlapping; the cut off value 1239853 had sensitivity of 72% and specificity of 38%.

The intensity values at 460nm also followed a similar pattern. Smokers were different from normal and pan chewers with a sensitivity and specificity of 100%. Normal and pan chewers showed sensitivity of 78% and specificity of 32% at cut off value of 1293550.

The present non-invasive in-vivo autofluorescence spectroscopy helps to differentiate smokers from normal with a significant difference. However further studies with more samples is necessary to identify the exact cause of decreased fluorescence in smokers, though we attributed it to the distortion of fluorescence as mentioned by Tsai et al [195]. The attempt to differentiate pan chewers from normal was also successful, since early changes with decreased fluorescent intensity compared to normal were noted.

Subsequently, biopsy samples were taken from the representative site as detected by autofluorescence. Only five patients from the each study group agreed for biopsy.

Histological comparison of the study group was compared with leukoplakia, sub mucous fibrosis, well differentiated and moderately differentiated squamous cell carcinoma and verrucous carcinoma.

After comparing the histological parameters, the presence of inflammatory cells was significant among the pan chewers when compared to

other study groups. This correlates to the possibility of increased progression to sub mucous fibrosis in pan chewers.

The dominant factor in the alteration of the mechanism of collagen synthesis is inflammation, especially chronic inflammation. The monocytes-macrophage sub group is now known to be capable of mediating matrix synthesis by messenger molecules like chemokines and growth factors and by synthesizing or activating degradative enzymes like proteinases and glycosidases. Among the growth factors, Transforming Growth Factor Beta (TGF-β) has been implicated as a key pro-fibrotic factor. However no growth factor or cytokine act in isolation and there is undoubtedly a complex, delicate array of interactions between the messenger molecules and their target cells [209].

Keratinization of mucosal epithelium is believed to be inter-related with the inflammatory cell infiltrate. In our study, the samples predominantly showed hyper-parakeratinization.

The epithelial thickness may depend on the tobacco habit and the location. An explanation for the epithelial hyperplasia is an adaptive response to local irritants to provide a greater degree of protection to the underlying connective tissue.

The collagen fibers were wavy in controls and in most of the pan chewers. Considering the amount and nature of collagen in the sub-epithelial region and deeper region, the haphazard and thickened arrangement of connective tissue seen in smokers along with lysis, correlates with the

connective tissue changes seen in the early stages of sub mucous fibrosis, where the collagen is thickened and seen as separate bundles [210].

The connective tissue production is permanent and there is no reversal of the condition even after the cessation of the habit. The bulk of the connective tissue consists of type I collagen and the formation does not appear to be related to excessive proliferation of fibroblasts.

In fact, smokers and pan chewers showed minimal fibroblasts. It has been shown that areca nut extracts suppress rather than stimulate the growth of cells in culture. Work done in UK has shown that the fibroblasts in oral sub mucous fibrosis are connected to collagen production rather than cell proliferation. It has been proposed that fibroblasts are functionally heterogeneous and play varied roles in any given normal/ diseased tissue.

Subsequently, to confirm to our histopathological findings, Transmission electron microscopy was done.

In smokers, Presence of numerous discontinuous bundles, few dense granules, There are few fibroblasts actively secreting collagen. There are increased bundles of collagen. The fibroblasts are elongated to spindle shaped, with elongated nucleus pushed to one end, numerous endoplasmic reticulum, extended cisternae, granules filled with proteinaceous material. There is presence of mast cells. The collagen bundles show prominent banding.

Our study also revealed the presence of epithelioid, spindle or elongated and stellate shaped fibroblasts in the connective tissue.

In accordance with literature, Mollenhauer and Bayreuther [208] described three distinct fibroblast cell forms in rat connective tissue that can be identified on the basis of their morphology. They can also be distinguished from one another by the amount and type of collagen synthesized.

4. *The F1 fibroblast is spindle shaped, highly proliferative and secretes low levels of type I and III collagen.*
5. *The F2 fibroblast is more epitheloid, less proliferative and synthesizes relatively more collagen.*
6. *The F3 fibroblast is a large stellate cell and the least proliferative, produces four to eight times more types I and III collagen than F1.*

According to these workers, F2 cells sequentially arise from F1 cells and F3 cells sequentially arise from F2 cells.

The lysis and fragmentation of collagen fibres was also considered as one of the change especially authenticated as an ultrastructural change in sub mucous fibrosis. The collagen fibrils were observed to be fragmented, some of them showed frayed and bent ends with apparent partial degeneration into amorphous material [28].

Binnie and Cawson [119] found an excess of fine, immature fibrils and inter-fibrillar matrix in sub mucous fibrosis. Rajendran et al (1986) also reported similar ultra-structural findings in sub mucous fibrosis.

Thus the ultrastructural changes of increased fibrosis in smokers, is in accordance with the findings of autofluorescence and histopathology and these changes are comparable to oral sub mucous fibrosis.

The response of tissue in relevance to smoking differs from pan chewing. In smoking, apart from the tobacco component, heat also acts as an additional source of insult and may aid in faster diffusion. The initial response of collagen fibres in smoking could be due to the synergistic effect of heat and tobacco. The collagen response would prevent the further diffusion, resulting in changes in epithelium and may thereby progress to leukoplakia and other changes.

In pan chewing, diffusion may be slow in comparison to smoking, thereby producing an initial, protective inflammatory response. The fibroblasts would initially produce collagen as a defensive reaction and subsequent insults would result in the morphologic change of fibroblasts and thus progress to sub mucous fibrosis.

Our study showed that smokers had connective tissue changes similar to sub mucous fibrosis more than that seen in pan chewers. Literature states that sub mucous fibrosis is more prevalent in pan chewers and smokers have increased tendency to develop leukoplakia, though both are caused by tobacco products.

Our study suggests that the initial connective tissue changes determine the progression of altered mucosa to a pre-cancer or cancer which is related to other complex interactions. Further studies are required to evaluate the significance of our hypothesis.

SUMMARY & CONCLUSION

SUMMARY AND CONCLUSION

In the present study the following salient features were found,

- *Although there are many fluorophores in the tissues, only the emission characteristics of tryptophan, collagen and NADH provided measurable variations.*
- *Our results showed distinct difference between normal mucosa and smokers.*
- *Significant difference was also found between pan chewers and smokers.*
- *However, we could also discriminate pan chewers from normal individuals.*
- *Although we attributed the decreased fluorescence intensity in smokers, is probably due to the distortion caused by dense fibrosis as advocated by Tsai et al or due to the conformational changes in the collagen molecules, further studies are necessary to find the exact reason for the decreased fluorescence.*
- *The intensity of smokers at 390nm is less when compared to normal and pan chewers, correlating with the findings of Tsai et al [195].*

Considering the histopathological parameters, the pattern of the sub-epithelial connective tissue, the presence of chronic inflammatory cells in the sub-epithelial connective tissue and deeper regions of connective tissue, presence of lysis of connective tissue was significant.

The ultrastructure of the fibroblasts among the study group, revealed the presence of epithelioid, spindle or elongated and stellate shaped fibroblasts in the connective tissue.

Our study showed that smokers had connective tissue changes similar to sub mucous fibrosis more than that seen in pan chewers.

Literature states that sub mucous fibrosis is more prevalent in pan chewers and smokers have increased tendency to develop leukoplakia, though both are caused by tobacco products.

The initial connective tissue changes could determine the progression of altered mucosa to pre-cancer or cancer. This transformation is related to complex interactions. A clear elucidation has to be obtained through molecular biological studies in the future.

Further studies are required to evaluate the significance of our hypothesis.

Smoking and pan chewing are preventable habits associated with considerable morbidity and mortality. Those who survive the ill-effects suffer personal consequences and become a considerable burden on the health care system. Even if patients cannot reverse their habits, modifications may reduce the risk. The awareness among the patients and the health care provider would facilitate early diagnosis of the disease.

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